Dysbiosis of the oral commensal microbiota drives inflammatory periodontal disease in the mouse model

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Thesis submitted to Queen Mary the University of London in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD)

July 2013

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List of Publication, Presentations & Prizes

Publications:-

Hajishengallis G, Liang S, **Payne MA**, Hashim A, Jotwani R, Eskan MA, et al. Low-Abundance Biofilm Species Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and Complement. **Cell Host Microbe 2011;10(5):497-506**

Presentations:-

Periodontal Diseases Gordon Research Conference
Davidson College
North Carolina, USA
July 2011

M A Payne, A Hashim, R P Darveau, A Alsam, M A Curtis
Comparison of Periodontal Bone Loss in a Germ Free and Specific Pathogen Free Mouse Model

Poster presentation

British Society for Oral and Dental Research (BSODR)
Sheffield University, UK
Spetember 2011

M A Payne, A Hashim, R P Darveau, A Alsam, M A Curtis
Role of the Commensal Microbiota in Porphyromonas gingivalis Induced Periodontitis

Oral presentation for Senior Colgate Prize

International Association for Dental Research (IADR) General Session

Iguaçu Falls, Brazil

June 2012

M A Payne, A Hashim, R P Darveau, A Alsam, M A Curtis

A Low-Abundance Biofilm Species Orchestrates Inflammatory Disease through the Commensal Microbiota

Oral and poster presentation for Unilever Hatton Prize

Prizes:-

Winner Senior Colgate Prize

Presented by British Society for Oral and Dental Research (BSODR)

September 2011

UK Representative in Senior (Basic Science) Unilever Hatton Prize

Presented by International Association for Dental Research (IADR)

June 2012

Acknowledgements

I would like to take this opportunity to express my sincere gratitude to Professor Mike Curtis without whose patience, support, understanding, friendship and guidance this work would not have reached fruition. You are a genuine polymath and inspiration.

I owe a huge debt of gratitude to Professor Francis Hughes for sparking my interest in the field of periodontal research and for his guiding hand as I took my first steps as a researcher and specialist clinician.

I wish to thank Dr Ahmed Hashim for getting me started in both animal research and associated microbiology, your infinite patience was hugely appreciated.

I need to give enormous thanks to Dr Joseph Aduse-Opoku for his guidance and support in trying to understand the complexities and intricacies of molecular biology. You are an unending font of knowledge and your dedication and commitment a lesson to all.

For unwavering support and for welcoming me to the group I wish to thank Dr Minnie Rangarajan, Dr Nikolay Paramonov and Juliet Ellwood. Your good cheer was a constant source of pleasure.

I would also like to thank Professor William Wade and Dr Hayley Thompson for their assistance in working up the methodology for the sequencing of our samples.

Dedication

On a personal note I want to dedicate this work to my soul mate and guiding light Sarah without whom none of this would have been possible. Your infinite patience and understanding is amazing and you keep me going when times get tough.

To my baby son Sam whose arrival during the course of this thesis was a moment of unparalleled joy. I hope that one day you will ask to read Daddy's book and that it might spark some interest in you to reach for and achieve your dreams. Always stay curious and remember the sky is not the limit.

An unmeasuarble debt of gratitude must go to my parents who have supported me throughout my endeavours. Your belief in me has always been appreciated and your unwavering backing of me beyond words. I think it was was worth the ride.

Abstract

Periodontal disease is a chronic inflammatory disease affecting the structures supporting the teeth. It results from the interaction between a microbial biofilm on the tooth surface and a de-regulated host response in the periodontal tissues of a genetically susceptible host. There are strong correlations between specific 'red complex' micro-organisms within the subgingival biofilm and disease. Dysbiosis, a deleterious shift in the relative abundance of components of the microbiota in disease, is a recognised property of microbiomes at other sites of the GI tract in chronic diseases.

Exploring dysbiosis in the oral commensal microbiota using a mouse model of periodontitis, we have shown that a 'red complex' organism (*Porphyromonas gingivalis*) caused significantly more periodontal bone loss in specific pathogen free (SPF) mice than controls and no bone loss in germ free (GF) mice. This confirms the oral commensal microbiota is fundamentally required for periodontal bone loss. In addition, low level colonisation of SPF mice with *P. gingivalis* led to qualitative and quantitative changes to the microbiota; dysbiosis. The oral commensal microbiota of the SPF mice was stable for our aging population of SPF mice and this led to increased alveolar bone loss with age.

Through a series of co-caging experiments we have shown that the oral commensal microbiota of different strains of mice was transmissible into GF mice and led to periodontal bone loss. We have also demonstrated that a dysbiotic oral commensal microbiota was transmissible into GF mice and led to increased periodontal bone loss.

In conclusion, the oral commensal microbiota is fundamental in the pathogenesis of periodontal disease in this mouse model. Moreover, it is dysbiosis of this oral commensal microbiota, brought about by *P. gingivalis*, that drives accelerated alveolar bone loss. We propose that *P. gingivalis* be considered as a keystone species.

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Chapter 5

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Introduction

1. Introduction

Periodontal disease is a chronic inflammatory disease that affects the tissues that support the teeth, namely the periodontal ligament, the root cementum and the alveolar bone. It is a major cause of tooth loss worldwide with debilitating consequences in terms of oral appearance and function.

Periodontal disease is a multifactorial disease but disease pathogenesis is initiated by a microbial challenge, in the form of a subgingival plaque biofilm on the surface of a tooth, and a de-regulated host inflammatory response in the periodontal tissues of a susceptible individual.

The nature of the microbial challenge has been extensively investigated and it has been shown that the microbiota undergoes an extensive change in composition in the transition from health to disease. There are very strong correlations between the amount and composition of the dental plaque biofilm and periodontal disease. Further investigations have shown that specific micro-organisms are strongly associated with periodontal disease. These micro-organisms tend to form complexes that are associated with severe and progressive disease and are termed the ‘red complex’.

Dysbiosis, or a shift in the relative abundancies of individual components of the microbiota compared with health, is now recognised as a property of microbiomes at other sites of the gastro-intestinal tract in several complex diseases such as inflammatory bowel disease, antibiotic associated diarrhoea, colo-rectal cancer and even obesity.

The aim of these studies was to define the commensal microbiome and explore dysbiosis of the oral microbiota in periodontal disease by means of a mouse model. Furthermore, the study aimed to examine how a known periodontal pathogen (*Porphyromonas gingivalis*) influenced the commensal microbiome and its effect on the pathogenesis of periodontal disease. Lastly, this study addressed the influence of mouse age, strain and genotype on the commensal microbiome and examined the transmission of both symbiotic and dysbiotic microbiomes into germ free mice and their influence on the development of disease.

1.1. Chapter 2

Chapter 2 consists of the background literature review to this thesis. It starts with considering periodontal disease as a whole with a brief review of the epidemiology, classification and risk factors for developing periodontal disease. There then follows a more detailed review of the pathogenesis of periodontal disease with focus on the areas that are further investigated in these studies.

The main theme of this work is the oral commensal microbiota and its alteration in disease. The next section details the microbiological aetiology of periodontal disease and the background to the methodology used in this work. A summary is presented of the current hypotheses that link plaque biofilms as the main aetiological agents in periodontal inflammation along with a detailed exploration of the plaque biofilm as a specific ecological entity. The concept of dysbiosis as a driver of disease in other parts of the human body is then explored and the idea of keystone species in disease pathogenesis.This concept is developed in relation to *Porphyromonas gingivalis* and its virulence in periodontal disease.

Finally, the use of animal models in periodontal research is reviewed with a focus on the mouse model and specifically the oral gavage model of experimental periodontitis used in these investigations. This includes a detailed analysis of the measurement of periodontal disease in this model.

The aims and objectives formulated from this background are then presented and these inform the experimental investigations that are presented in this thesis.

1.2. Chapter 3

In Chapter 3 the experimental materials and methods are outlined with specific focus on the mice used in these studies and the establishment of 2 genetically identical colonies of mice in specific pathogen free and germ free conditions. The details of the knockout mice are also described.

The techniques used for preparation and delivery of the oral inocula are detailed along with the microbiological methods used for oral sampling of the mice. There then follows an outline of the different techniques used to identify the oral commensal microbiota and specific components of this microbiome (*P. gingivalis*).

Finally, there is an explanation of the analysis of alveolar bone loss and histological analysis for osteoclasts. The Chapter concludes by outlining the statistical basis for analysing the results.

1.3. Chapter 4

In Chapter 4 the main results from these studies are presented. The initial data relate to detailed definition of the commensal microbiota in the C3H mouse, the effects of aging and antibiotics on this microbiome and the efficiency of its transmission into a germ free mouse.

Data is then presented showing how a periodontal pathogen (*P. gingivalis*) induces dysbiosis in this commensal microbiota and how that translates into increased alveolar bone loss. The results of the transmission of this dysbiotic microbiota into a germ free mouse are then presented.

Finally the influence of mouse strain on the nature of the commensal microbiota and the effects on this microbiome of transgenic mice with defective immune response is presented. Data showing transfer of a mouse strain dependant microbiota and a genetic knockout dependant microbiota into germ free mice and the effects on periodontal bone loss is then presented.

1.4. Chapter 5

In chapter 5 the results are presented in the context of the existing body of research and development of the limitations into areas for future research. Specifically, the stability of the commensal microbiota is discussed and the differences in composition that were found when comparing culture and non-culture dependant techniques highlighted. The importance of the part of the microbiome that has yet to be fully defined and how the role this may play in the pathogenesis of periodontal disease is then speculated upon.

The concept of the keystone hypothesis of pathogenesis is supported by the data presented and the suggestion that these data may offer a way of integrating the various plaque hypotheses proposed. Critical focus on the oral gavage model used and, specifically on the 'superinfection' of *P. gingivalis* that is used, follows and the implications of this.The data presented also offers an insight into the role of the neutrophil in the pathogenesis of periodontal disease.

The final point that is developed is the idea of the transmissibility of the oral commensal microbiota from an SPF mouse into a germ free. The results suggest that this is possible in terms of a 'normal' and a dysbiotic microbiota.

1.5. Chapter 6

In this chapter the development of future lines of investigation are consolidated. Suggestions as to how one might investigate the components of the commensal microbiota that are only present in small numbers with a view to assessing their significance are discussed. Also how one might further support the concept of *P. gingivalis* as a keystone pathogen and how the transmission of a *P. gingivalis* induced dysbiotic microbiota might be further investigated are outlined. Finally, a critique of the use of this mouse model follows and suggestions as to how modifications might be needed for future research needs

Chapter 2

Literature review and background

Periodontal disease is a chronic inflammatory disease that affects the tissues that support the teeth, namely the periodontal ligament, the root cementum and the alveolar bone.

2.1. Prevalence

In the 2009 Adult Dental Health Survey[1] carried out in England, Wales and Northern Ireland more than 54% of adults aged over 55 years showed gingival bleeding; a sign of gingivitis. Gingivitis and periodontitis (periodontal disease) are considered a continuum of the same disease process[2]. Overall the survey found that 45% of over 55 year olds had periodontal pocketing (a major sign of periodontal disease) with 7% having moderate pocketing (6-9mm) and 1% severe pocketing (>9mm). Similarly, 21% of the same groups of adults had loss of attachment (LOA) of periodontal tissues to a moderate level (>6mm) and to a severe level (>9mm) in 4% of individuals. The report also commented on the trends in disease prevalence and noted that 'no decline in prevalence was observed' between the last deciennial survey. Indeed, for moderate to severe periodontal pocketing there was an increase in overall prevalence from 6 to 9%.

In the United States of America there are similar regular surveys of health; the National Health and Nutrition Examination Survey (NHANES)[3]. The latest data from 2004 found that 8.52% of adults aged 20-64 years had periodontal disease with 5.08% having moderate to severe disease. In the older age groups (>65 years) the prevalence was 17.2% with moderate to severe disease in 10.58%.

The prevalence of periodontal disease also appears to be increasing in other countries in the developed world. For example in similar health surveys[4] carried out in Germany in 1997 and 2005 the prevalence of moderate pocketing (4-5mm) increased from 32.2% to 52.7% and 39.7% to 48% in younger adults (35-44 years) and older adults (65-74 years) respectively. Similar increases were found with severe pocketing increasing from 14.1% to 20.5% in the younger ages and 24.4% to 39.8% in the older population.

Despite a number of global surveys looking at the prevalence of periodontal disease the pattern is far from clear. The situation is compounded by variations in methodology of assessing and categorising disease status. At a global level, severe periodontal disease is suggested to affect 5-15% of the adult population[5].

In addition, it is likely to become an increasing burden especially in developing countries. Global population expansion is predicted to increase by 33% in the next 40 years from the current 7 billion people, a large part of this increase occurring in the poorer countries[6 7]. Individuals from lower income groups in these countries tend to be associated with increased risk factors for periodontal disease[8 9] and more severe periodontal disease[10, 11].

2.2. Classification of Periodontal Disease

Classification of Periodontal Diseases has historically been challenging because of its multifactorial aetiology and highly varied manifestations. The current classification is highly complex[12-15] and detailed and is still far from ideal. The simplest way of visualising the classification is detailed in Table 2.1[16].

Disease Classification	Sub-classification
Gingival diseases	Plaque induced
	Non-plaque induced
Chronic periodontitis	Localised
	Generalised
Aggressive periodontitis	Localised
	Generalised
Periodontitis as manifestation of systemic disease	
Necrotising periodontal disease	
Abscesses of the periodontium	
Periodontitis associated with endodontic lesions	
Developmental/Acquired conditions	

Table 2.1 Summary of the classification of Periodontal diseases[16].

2.2.1. Chronic periodontal disease

Chronic and Aggressive periodontitis appear to be different manifestations of periodontal disease and have distinct features (Table 2.2 & 2.3).

Features of Chronic Periodontitis
Increased prevalence in adults (but can affect children/adolescents)
Destruction of periodontal tissues is consistent with presence of local factors
Subgingival calculus commonly found
Associated with variable microbial pattern
Slow-moderate rate of progression but may be superimposed periods of more rapid destruction
Further classified on extent (localised and generalised) and severity (mild, moderate or severe)
Can be associated with local predisposing factors
Can be modified by and/or associated with systemic disease
Can be modified by other factors e.g. cigarette smoking and emotional stress

Table 2.2 Features of Chronic periodontitis[16].

2.2.2. Aggressive periodontal disease

Aggressive periodontitis can exist in localised or generalised forms and has a number of common features (Table 2.3) that are associated with the disease and a number of secondary features (Table 2.4) which may be present but are not universal.

Common features of aggressive periodontitis
Individuals are otherwise clinically healthy
Rapid attachment loss and bone destruction
Familial aggregation

Table 2.3 Common features of aggressive periodontitis[16].

Secondary features of aggressive periodontitis
Amount of plaque inconsistent with severity of tissue breakdown
Elevated levels of specific pathogens (*A. actinomycetemcomitans* and *P. gingivalis*)
Phagocyte abnormalities
Hyperresponsive macrophage phenotype with elevated PGE_2 & IL-1β response to bacterial LPS
Progression of tissue destruction may be self-arresting

Table 2.4 Secondary features commonly (but not universally) associated with aggressive periodontitis[16].

2.2.2.1. Localised aggressive periodontitis

Aggressive periodontitis is further sub-classified based upon number of sites affected, <30% is termed localised aggressive periodontitis (LAP). There are enough distinguishing features of LAP for it to be considered as a separate disease entity (Table 2.5).

Common features of LAP
Onset around puberty
Localised involvement of 1^{st} molars & incisors with interproximal bone loss on more than 2 permanent teeth, one of which is 1^{st} molar, and involving no more than 2 teeth other than 1^{st} molar or incisors
Robust serum antibody response to infecting agents

Table 2.5 Common features of localised aggressive periodontitis (LAP)[16].

2.2.2.2. Generalised aggressive periodontitis

If there is greater than 30% involvement aggressive periodontitis is termed generalised aggressive periodontitis and individuals with this form of the disease share some different common features (Table 2.6)

Common features of GAP
Usually affects individuals under 30 years
Generalised interproximal bone loss affecting minimum of 3 permanent teeth other than 1st molars or incisors
Episodic nature of tissue destruction
Poor serum response to infecting agents

Table 2.6 Common features of generalised aggressive periodontitis[16].

2.3. Risk factors for periodontal disease

Periodontal diseases are multi-factorial in nature and manifest a wide range and degree of signs and symptoms[8, 10].

2.3.1. Smoking

Cigarette smoking has been shown to be a major risk factor for periodontal disease. In early studies involving Swedish Army conscripts[17], a cohort with high levels of oral hygiene, data showed that in smokers there was an increased risk of gingivitis and in a later study[18] decreased alveolar bone height. The relative risk of smoking for moderate to severe periodontal disease has been estimated to be 2.7 when comparing smokers with non smokers[19].

In addition to increased risk of periodontal disease and the severity of the disease there is also evidence that the response to treatment is poorer. Systematic reviews of treatment outcome parameters show that in smokers there is less reduction in probing depth after treatment[20]. In addition, the majority of clinical trials show an increased gain in Clinical Attachment Levels (CAL) in non smokers following treatment[21] and that quitting is beneficial to treatment outcome. Smoking is also a predictor of further tooth loss during periodontal maintenance therapy[22].

The effect of smoking on the periodontium appears to be primarily through its effects on the neutrophils and the microvasculature[23]. Neutrophils are impaired in terms of transmigration and function and may also be responsible for producing proteases which lead to tissue destruction. In addition, smoking can affect the subgingival micro flora. Studies[24] have found higher proportions of periodontal pathogens such as *Aggregatibacter actinomycetemcomitans*, *Tannerrella forsythia* and *Porphyromonas gingivalis* in smokers when compared to non smokers. Similarly 8 members of the orange and red complex micro-organisms (see section 2.5.5. & Figure 2.9. for explanation) were found to be present in higher numbers in smokers when compared to ex or non smokers and these were also found to colonise shallower sites[25].

2.3.2. Socio-ecomomic factors

Low socio-economic status has been proposed as a risk factor for periodontal disease. In the USA, based on the data in the NHANES III studies, statistical modelling has shown that as socio-economic status decreases there is an increase in gingival bleeding and attachment loss[26]. Similarly in a Swedish study[27] there was a correlation between differences in income levels and severe bone loss with an odds ratio of 8.46.

However, the multifactorial nature of periodontal disease means that inevitably the effect of socio-economic status is more complex with many confounding issues. More detailed analyses of these effects have been attempted[28, 29].

2.3.3. Nutritional factors

The links between nutritional levels and association with periodontal disease has been investigated. For example as part of the third National Health and Nutrition Examination Survey in the USA (NHANES III) it was found that low calcium intake was associated with increased risk of periodontal disease among young males and females and older males[30]. The association of specific nutrients with periodontal disease has been extensively reviewed[31] but the associations between specific nutritional deficits and periodontal disease are not strong enough to recommend the use of dietary supplements[32, 33]. Further studies are required to elucidate any links between nutritional deficiencies and periodontal disease.

2.3.4. Psychological factors

Stress has been proposed as a risk factor for developing periodontal disease. In one early study, 1426 subjects with periodontal disease, which was verified through clinical indices of disease, filled out 5 psychosocial instruments designed to measure life events, daily strains, hassles and uplifts, distress and coping mechanisms[34]. They found that financial strain was significantly associated with greater attachment loss and alveolar bone loss and further analysis showed individuals with high emotion-focussed coping strategies (termed inadequate coping) had even higher risk of more severe attachment loss than individuals with problem-based coping strategies. In addition, salivary cortisol levels were highest in individuals with severe periodontitis experiencing high levels of financial strain and with inadequate coping.

In a case control study of 100 dental patients it was found that periodontitis was significantly associated with negative life events, the number of negative life events and unemployment as well as other risk factors such as high levels of dental plaque and smoking[35]. They concluded that psychosocial factors and oral health risk behaviours cluster together as important determinants of periodontitis.

Mechanisms for the role of psychosocial stress in the development of periodontal disease have been proposed (Figure 2.1). Chronic stress stimulates the hypothalamus-pituitary-adrenal (HPA) axis and this leads to the secretion of cortisol (which stimulates immune responses), catecholamines (which stimulate the autonomic nervous system) and neuropeptides such as substance P. Release of these substances can directly influence the composition of the plaque biofilm, especially the pathogenic species, or may influence the inflammatory response. The net result could be increased infection and inflammation[36].

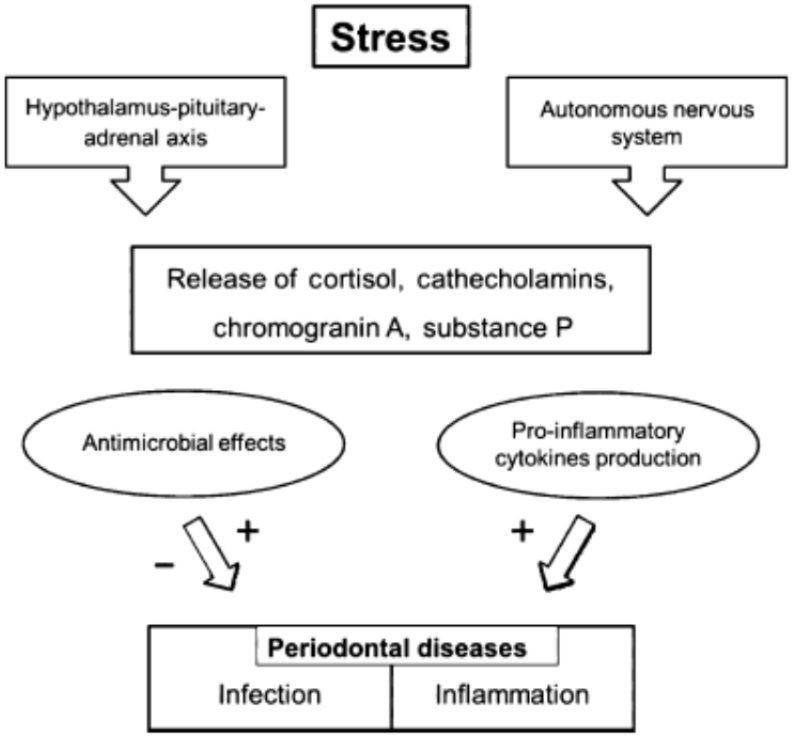


Figure 2.1 Mechanisms through which stress can influence periodontal disease status through stimulation of the hypothalamus-pituitary-adrenal axis and release of stress hormones that can directly influence the composition of the subgingival biofilm or host inflammatory response. From Akcali et al[36].

2.3.5. Systemic factors

Probably the most explored area of a link of systemic disease with periodontal disease is in diabetes mellitus. Initial data were somewhat conflicting but the relationship became more fully understood following a cross-sectional study of a group of Pima Indians who have a high prevalence of type II diabetes[37-39]. This group live in a reservation on the Gila River in Arizona USA and have the world's highest reported incidence of type II diabetes affecting 40-50% of the population. Diabetic status of the 1342 subjects was assessed and was found to be associated with increased prevalence and severity of destructive periodontal disease. The odds ratio of being diabetic and increased risk of periodontal disease was 2.81 if attachment loss was used as the measure of periodontal disease or 3.43 if bone loss was the measure. Other cross-sectional studies in other groups have found similar links of type II[40] and type I[41] diabetes with increased risk of periodontal disease.

Systematic reviews have attempted to combine the results from the multitude of studies[42]. Despite a large degree of heterogeneity between studies it was estimated that there was significantly greater mean attachment loss of 1mm in diabetics compared to non-diabetics and increased mean probing depths of 0.46mm.

Longitudinal studies have also highlighted the links between diabetes and the risk of periodontal disease and these have also shown the importance of glycaemic control. In follow up studies[43] in the Pima Indians glycosylated haemoglobin (HbA1c) was used as the long term measure of diabetic control and this was compared with alveolar bone loss as a measure of the severity of periodontal disease. It was found that in the 359 subjects poor diabetic control was associated with an odds ratio of 11.4 for increased risk of periodontal disease whereas in the better controlled group the odds ratio was 5.3. The conclusion was that poor glycaemic control was an increased risk for alveolar bone loss and more severe progression of disease.

Other workers have also shown the importance of glycaemic control in the risk of and severity of periodontal disease. In a group of non smoking type I diabetic patients studied in Glasgow, UK[41] the HbA1c levels were assessed and compared to levels of severe periodontal disease (defined as ≥ 6mm attachment loss on ≥ 1 tooth). It was found that the odds ratio for type 1 diabetes and severe periodontal disease was 1.35 and in poorly controlled diabetics was 1.58.

Data also supports the concept of a bidirectional relationship with diabetes and periodontal disease i.e. the increased risk of developing diabetes if one suffers from periodontal disease. The first data to suggest this relationship emerged from a follow up to the first National Health and Nutrition Examination Survey in the USA (NHANES I)[44]. Over 9000 individuals that had participated in a dental assessment in NHANES I were followed up at least once within a 19 year period. Periodontal disease was assessed by the periodontal index (0=no disease up to 5=severe disease) and diabetic status was either self reported, a death certificate where diabetes was listed or a stay in a health care facility where diabetes was on the discharge summary. In health and mild periodontal disease (indices 1-2) the odds ratio of developing diabetes was not elevated but in severe periodontitis (indices 3-5) the odds ratio of developing diabetes was 1.50-2.26.

Longitudinal studies in other geographic populations support this bidirectional relationship. In Pomerania, Germany nearly 3000 individuals that were initially diabetes-free were assessed for severity of periodontal disease and followed up after 5 years where glycosylated haemoglobin levels were also assessed as a measure of the development of diabetes mellitus. When comparing individuals with the highest and lowest severities of periodontal disease it was found that severe disease was associated with a fivefold increase in the development of diabetes at 5 years.

So it would seem that diabetes mellitus (both type I and II) are risk factors for periodontal disease specifically, increases in the prevalence, severity, extent and progression of the disease. The evidence supports the idea that poor glycaemic control is associated with more severe periodontal disease and that there is a bidirectional relationship with individuals with existing periodontal disease being at increased risk of developing diabetes[45].

2.3.6. Genetic factors

The influence of the genetics of the individual as a risk factor for periodontal disease emerged from twin studies looking at monozygotic and dizygotic twins reared together and separately. In these studies a significant genetic component was identified for gingivitis, probing depth, attachment loss and plaque with estimates that 38-82% of the population variances for these measures were attributed to genetic factors[46]. These were followed up with comparison of panoramic radiographs which showed that between and within group bone levels were similar for each group[47]. In a later follow up study[48] the same group found that the similarity between probing depth, attachment loss and gingival indices were more similar for monozygotic twins than dizygotic twins. It was estimated that adult periodontitis was around 50% heritable.

Other workers have tried to establish the mode of inheritance. For example aggregation of aggressive disease in a specific cohort of black families[49] was determined as autosomal dominant. Other groups have shown that there are aggregations of aggressive periodontitis among certain families with affected pedigree members reaching 40-50%[50]. There does however remain debate about the exact manner in which periodontal disease may be inherited.

Further evidence for genetics as a risk factor for periodontal disease comes from genetic polymorphisms and periodontitis. These have been reviewed recently[51, 52] but the general findings are that gene polymorphism studies cannot distinguish between aggressive and chronic periodontitis. Commonly investigated gene clusters which have been studied for polymorphisms include interleukin 1 (IL-1), tumour necrosis factor (TNF), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10) and pattern recognition receptors such as CD-14. The evidence suggests that genetic polymorphisms in IL-1, IL-6, IL-10, vitamin D receptor and CD-14 may play a role in chronic periodontitis but these associations are usually restricted to specific ethnic or racial groups[45].

2.3.7. Tooth factors

Various aspects of tooth anatomy and position have been associated as risk factors for periodontal disease. These include enamel projections[53, 54] and pearls[55] in the furcation, misaligned and crowded teeth[56-58]. Occlusion as a risk factor for periodontal disease is a more contentious area with some authors believing that occlusal trauma or discrepancies are a risk factor for periodontal disease[59] with other groups believing it is the underlying inflammation that is the driver of periodontal disease and not occlusal trauma per se[60]. Iatrogenic risks include poorly placed restorations with ledges and overhangs[61] and subgingival crown margins[62] all associated with increased risk of periodontitis.

2.3.8. Microbial factors

The majority of periodontal diseases are initiated by micro-organisms that inhabit a plaque biofilm on the surface of a tooth[63, 64]. These will be discussed in detail in section 2.5.

2.4. Pathogenesis of periodontal disease

The pathogenesis of periodontal disease involves a complex interplay between plaque bacteria and a susceptible host[65]. Ultimately inflammation is produced which leads to breakdown of periodontal ligament fibres, infection and loss of alveolar bone; an overall loss of clinical attachment level (CAL).

2.4.1. Histological changes in periodontal disease

The classical view is that inflammation of the gingival tissues, gingivitis, leads to inflammation of the periodontal tissues, periodontitis, although this may not always be the case. The histological changes within the tissues have offered an insight into the pathogenesis of periodontal disease.

The progression of periodontal disease has been classically described in 4 stages based on histology; initial lesion, early lesion, established lesion and advanced lesion[66]. In reality these stages may not be distinct entities in their own right or discernable from each other. They may be more correctly thought of as an overlapping continuum of the different histological features that constitute developing periodontal disease.

In health there is no breach of the oral epithelium with the junctional epithelium intact. The epithelium overlies connective tissue that is well ordered with densely packed and organised collagen fibres. There are sentinel neutrophils that patrol the connective tisue and act as a first line of defence against any invading micro-organism or antigens (Figure 2.2).

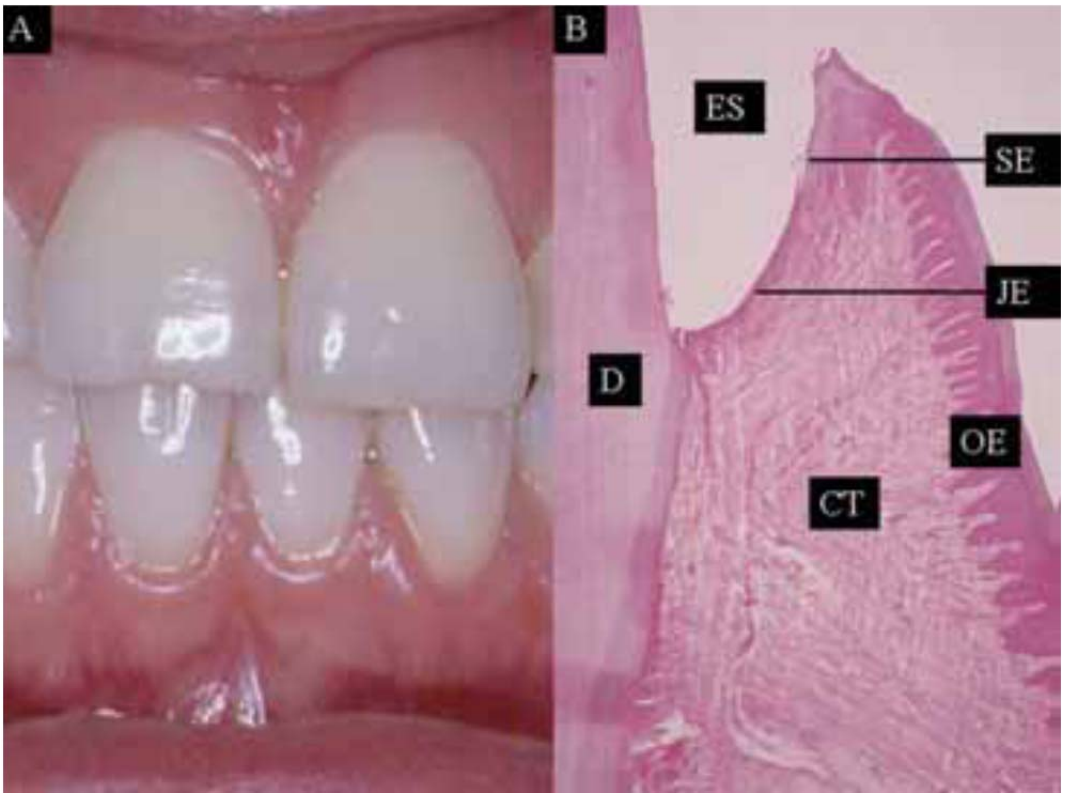


Figure 2.2 Healthy gingivae with clinical appearance (A) and histological appearance (B) showing intact junctional and sulcular epithelium (JE & SE respectively) continuous with the oral epithelium (OE) and overlying an ordered dense connective tissue (CT) in conjunction with the root surface dentine (D). The space occupied by the enamel prior to decalcification is the enamel space (ES). From Preshaw et al [65].

With the accumulation of plaque at the gingival margin there is a direct bacterial challenge to the periodontal tissues and an inflammatory/immune response is initiated. Bacterial antigens activate the sentinel neutrophils and a more robust innate response is initiated. Greater numbers of inflammatory/immune cells are recruited into the tissues (neutrophils, plasma cells, macrophages and lymphocytes) and this accumulation of defence cells disrupts the ordered structure of the connective tissue with resultant breakdown. There is dilation of the blood vessels and at a macroscopic level oedema and erythema of the tissues (Figure 2.3).

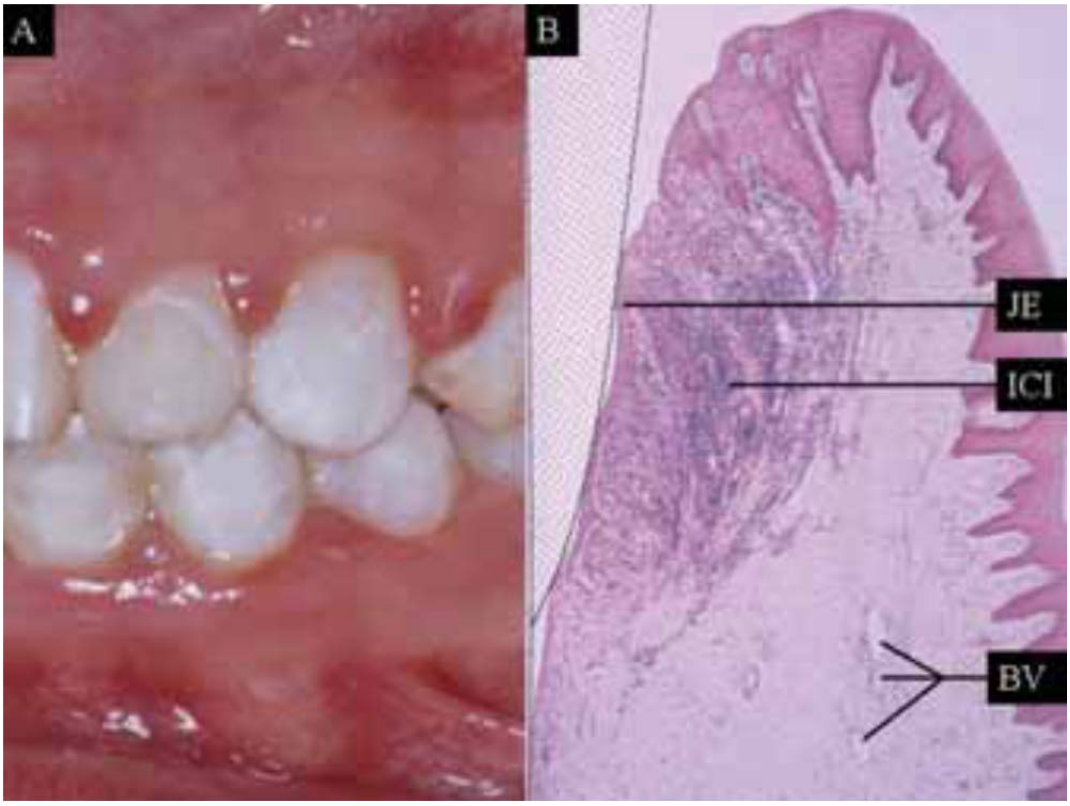


Figure 2.3 The clinical appearance of gingivitis (A) with oedema and erythema and histological changes (B) with dilation of blood vessels (BV) and inflammatory/immune cell infiltrate (ICI) into the connective tissue and disrupting the junctional epithelium (JE). From Preshaw et al[65].

Progression into periodontal disease is marked by apical proliferation of the lesion as a result of further breakdown of the connective tissue due to the chronic irritation of the plaque biofilm. The lesion becomes more widespread with infiltration of inflammatory/immune cells extending further apically and laterally to include the periodontal ligament fibre and the alveolar bone with consequent destruction of these tissues (Figure 2.4).

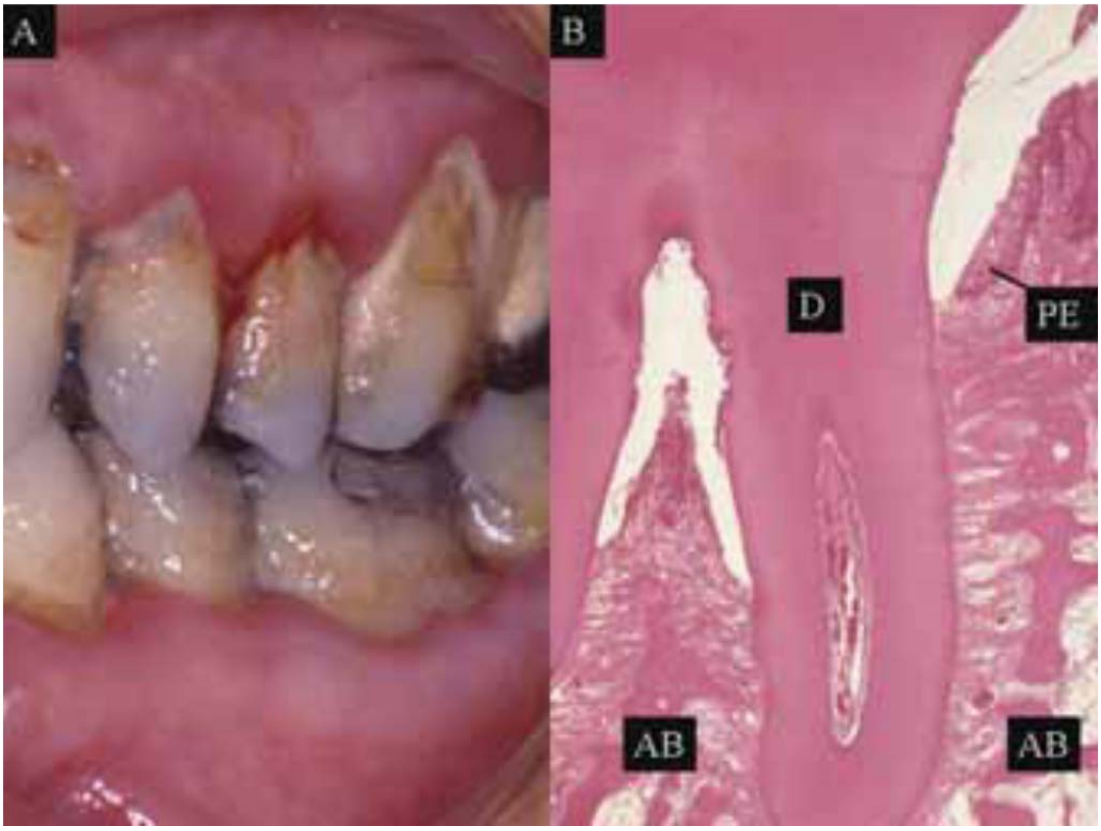


Figure 2.4 The clinical appearance of established periodontitis (A) with gross accumulations of plaque, increased pocketing, inflammation, oedema and bleeding of the tissues. The histological appearance (B) reflects more widespread involvement of the tissues with loss of attachment of the periodontal ligament fibres from the root surface dentine (D) and apical migration of the junctional epithelium to form a pocket lined by pocket epithelium (PE). There is loss of alveolar bone (AB). From Preshaw et al[65].

2.4.2. Innate host response response in health

As the plaque biofilm accumulates and matures, microbial peptides and antigens are released into the periodontal tissues via the ulcerated junctional epithelium. As can be seen by the histological changes these antigens are able to induce a robust host inflammatory/immune response in the periodontal tissues.

The primary cell type in the innate response is the neutrophil. These cells are able to migrate from the vasculature into the gingival tissue and ultimately into the gingival crevice. This transmigration requires the co-ordinated expression of intercellular adhesion molecules (ICAMs), E-selectin (ELAM-1) and chemokines including interleukin-8 (IL-8)[67, 68]. These neutrophils form a physical barrier adjacent to the plaque biofilm from where they are able to co-ordinate the response of other immune cells types[69]. This transit of neutrophils through the periodontal tissue is continuous and is a function of the constant microbial challenge even in health (Figure 2.5). More recently it has been established that some of the components of this neutrophil recruitment machinery may be expressed in the absence of a microbial challenge. Immunohistological investigations of the periodontal tissues of germ free mice have demonstrated the presence of neutrophils in the tissues and chemokine expression by the junctional epithelium[70].

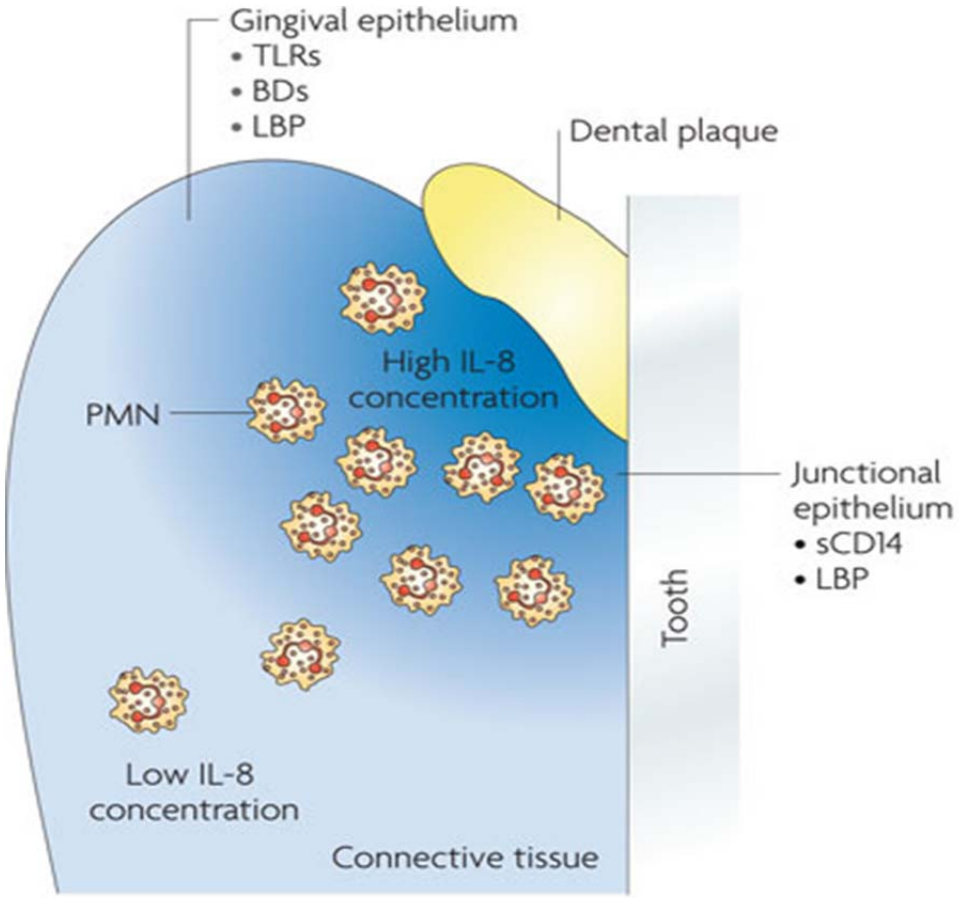


Figure 2.5 In health there is a transit of neutrophils trough the gingival tissues facilitated by the co-ordinated expression of innate mediators such as IL-8. In addition the gingival tissues express a number of innate host defence mediators that include toll-like receptors (TLRs), β-defensins and lipopolysaccharide binding protein (LBP) and there is expression of CD14 and LBP in the junctional epithelium. From Darveau R P. Nature Reviews in Microbiology[71].

Human β-defensins are antimicrobial peptides that are expressed in the periodontal tissues[72] which have the ability to disrupt the membrane integrity of a broad spectrum of micro-organisms. They are present in 3 forms, human beta defensins (hBD) 1, 2 and 3 and are expressed in both healthy and diseased individuals.

CD-14 is an important receptor for bacterial lipopolysaccharide (LPS) which is expressed by monocytes/macrophages and neutrophils. It is present as a membrane-bound form (mCD-14) and a soluble form (sCD-14)[73]. Investigations[73] have shown that sCD-14 levels in the GCF of individuals with untreated periodontitis are variable but that higher levels of sCD-14 and with more sites containing sCD-14 had fewer deep pockets implying a pivotal role for sCD-14 in the control of bacterial induced periodontitis. In addition, levels of expression of mCD-14 have been found to be increased in healthy gingival biopsies when compared to diseased tissue biopsies[74].

Lipopolysaccharide binding protein (LBP) is an acute phase reactant mainly derived from the liver whose plasma levels rise in response to inflammation induced by Gram negative bacteria. This protein plays an important role in neutralising LPS from Gram negative bacteria and can activate further cellular responses through induction of endogenous IL-1, TNF-α and by the LBP-LPS complex binding to mCD-14. The expression of this protein has been found to be increased in gingival biopsies of healthy controls compared to healthy tissue biopsies from periodontitis affected individuals or biopsies of periodontal pocket tissue[75]. This expression may offer protection from inflammation induced by Gram-negative LPS. Soluble CD-14 and LBP play a crucial role in the clearance of bacterial components.

Toll-like receptors (TLRs 1-9) are also expressed by the gingival tissues and these will be discussed further (section 2.4.4.3.).

2.4.3. The cytokine response in healthy periodontal tissues

The persistent oral commensal microbiota in direct contact with the junctional epithelium induces an innate protective response in the underlying periodontal tissues. The layers of the gingival epithelium in direct contact with the commensal plaque biofilm produce IL-8[76] and β-defensins[77]. This facilitates the transit of neutrophils through the gingival tissues.

Another important cytokine in the tissue homeostasis in periodontal health is interleukin 1 beta (IL-1β). The levels of IL-1β are higher in the gingival tissues in conventionally reared specific pathogen free (SPF) mice than in germ free (GF) mice indicating that it is colonisation with an oral commensal microbiota that modulates expression of this cytokine at an mRNA and protein level[78]. However, even in the germ free mice IL-1β is still expressed (at lower levels) with the implication being that it is involved in host homeostatic mechanisms independent of bacterial colonisation.

Other cytokines normally associated with a pro-inflammatory state (in addition to IL-1β[79, 80]) are also expressed in the gingival tissues of healthy individuals (as well as increased expression in diseased individuals). These include tumour necrosis factor alpha (TNF-α)[81, 82] and prostaglandin E_2 (PGE_2)[83]. The purpose of the expression, albeit in low levels, of these normally pro-inflammatory cytokines is to maintain tissue homeostasis in the periodontal tissues under the constant challenge from an oral commensal microbiota.

2.4.4. The shift into the disease state

One of the major conceptual changes in understanding the pathogenesis of periodontal disease was based on the discoveries that the host contributes to the breakdown of the tissues[84] (summarised in Figure 2.6).

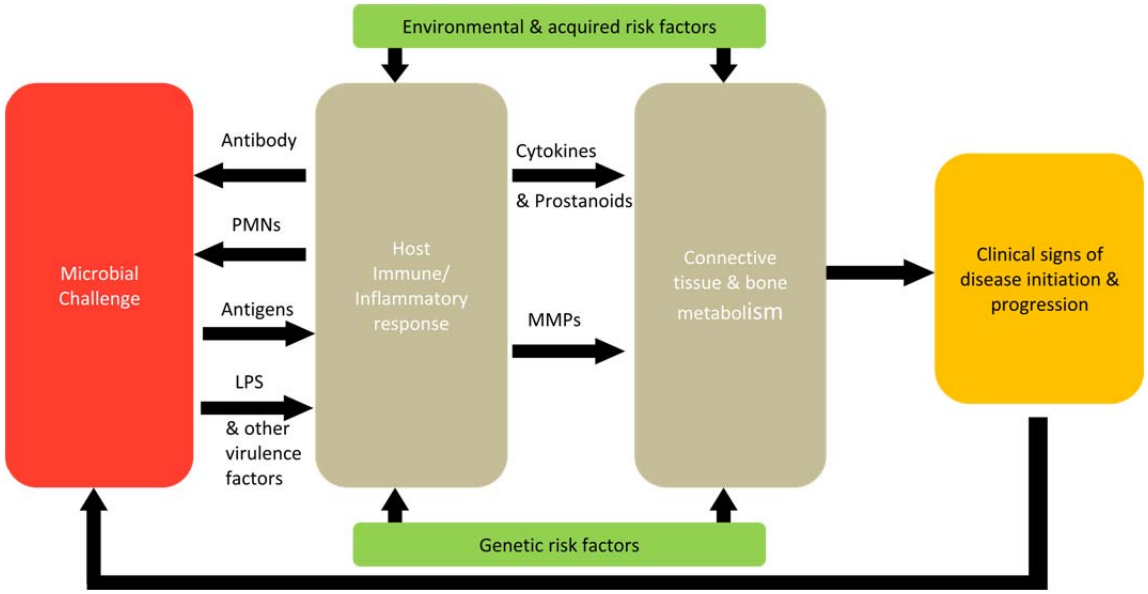


Figure 2.6 Summary of the pathogenesis of periodontal disease showing the process is initiated by a microbial challenge in the form of a plaque biofilm which produces various disease inducing factors (Antigens, LPS, virulence determinants) that interact with the host inflammatory/immune response. This interaction feeds back in an attempt to control the microbial challenge (antiboby, neutrophils) and ultimately leads to tissue damage through the release of cytokines and tissue proteinases (MMPs) and the signs and symptoms of disease. The development of disease then modifies the microbial challenge through micro-environmental changes. The overall disease process is modified by environmental, acquired and genetic risk factors. From Page and Kornmann[85].

2.4.4.1. Alveolar bone loss

Alveolar bone homeostasis is regulated through osteoclast differentiation via a signalling pathway that involves Receptor-Activator of Nuclear factor Kappa B Ligand (RANKL) and its antagonist osteoprotegrin (OPG)[86]. RANKL is expressed by a number of cell types (namely osteoblasts, periodontal ligament fibroblasts, human gingival fibroblasts and T & B lymphocytes) and it binds the receptor RANK which is expressed on osteoclast precursors. This ligand-receptor binding induces the differentiation of precursor cells into osteoclasts[87]. OPG is a soluble protein that binds to RANKL and prevents the RANK-RANKL interaction that initiates osteoclast differentiation from precursor cells. Ultimately, it is the ratio of RANKL/OPG in the tissues that determines whether there is net bone resorption (if RANKL predominates) or bone formation (if OPG predominates) (Figure 2.7). The levels of RANKL are increased by inflammation and specifically pro-inflammatory cytokines such as IL-1β and TNF-α whereas the levels of OPG are influenced by transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs)[88].

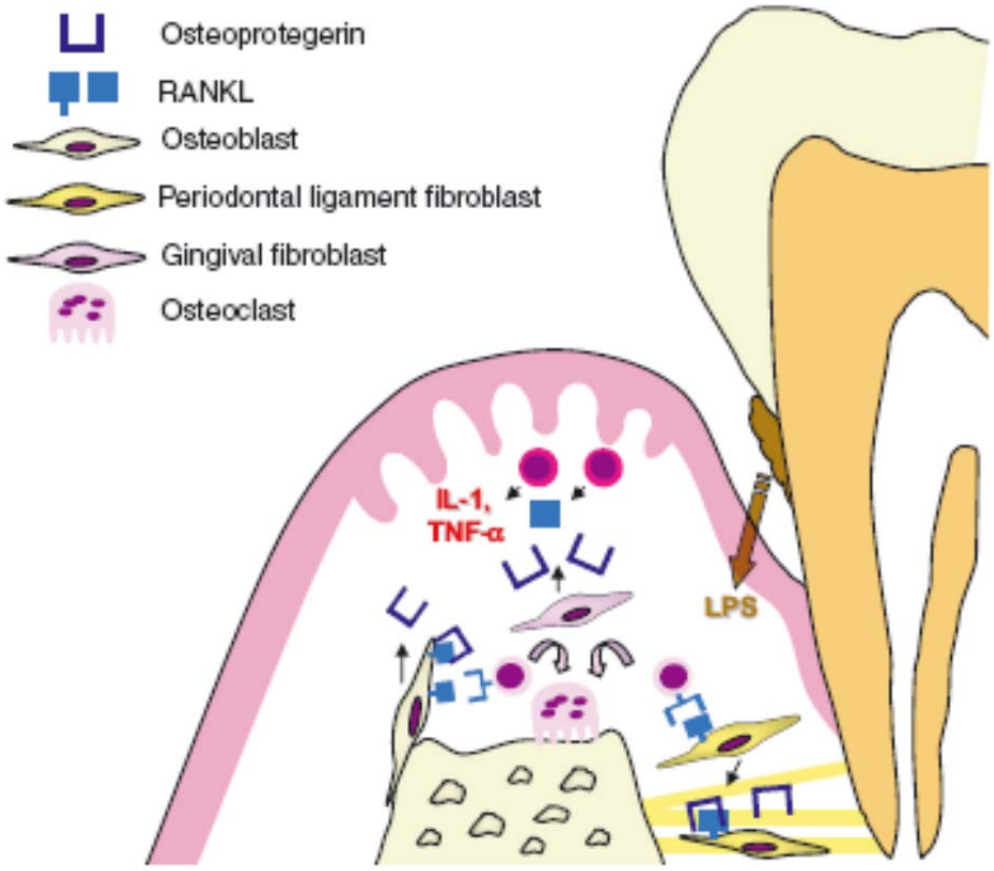


Figure 2.7 Alveolar bone levels controlled by the ratio of RANKL/OPG in the periodontal tissues. RANKL expressed by osteoblasts and fibroblasts binds RANK in response to inflammation (via IL-1β and TNF-α) and facilitates differentiation and activation of osteoclasts and bone resorption. OPG is induced by TGF-β and BMPs and binds the 'free' RANKL preventing binding to RANK on osteoclast precursors thus preventing the osteoclast differentiation pathway being activated. From Hagasawa et al[87].

2.4.4.2. Tissue breakdown due to host derived cytokines

A number of inflammatory mediators produced by the host have been implicated in the tissue destruction that accompanies periodontal inflammation. The majority of the evidence for their role in the pathogenesis of periodontal disease is derived from animal models, especially the use of transgenic mice that have mutations for genes that regulate specific mediators.

The role of interleukin 1 (IL-1α & IL-1β) in the pathogenesis of periodontal disease has been investigated in the macaque primate[89]. In these animals induction of periodontal disease with a silk ligature tied around the teeth was accompanied by a front of inflammatory cells that progress towards the alveolar bone and produce bone resorption. Soluble receptors to IL-1 and TNF injected into the tissues inhibited ~80% of the recruitment of the inflammatory cells to the proximity of the bone compared to controls. There was also a 67% reduction in formation of osteoclasts and 60% reduction in bone loss compared to control sites not treated with the soluble receptors[90]. Inhibition of IL-1 and TNF led to decreased alveolar bone loss, suggesting that it is through these cytokines that a significant component of the pathologic process is mediated. In follow up studies it was found that inhibition of these mediators with soluble antagonist led to 51% reduction in connective tissue loss and reduction in loss of alveolar bone height by 91%[91]. Indeed, over-expression of IL-1β (in transgenic mice that over-express IL-1β in the gingival epithelium) has been shown to produce tissue break down and bone loss in a manner that resembles periodontal disease, even in the absence of an infecting micro-organism[92].

Interleukin 6 (IL-6) and interferon gamma (IFN-γ) have also been implicated in the pathogenesis of periodontal disease from studies using the mouse model. In one study[93] using the oral gavage model of experimental periodontitis, a number of different genetic knockout mice were inoculated with *P. gingivalis*. Severe combined immunodeficient mice (SCID), mice with no T or B lympohcytes, exhibited significantly less alveolar bone loss following challenge with *P.gingivalis* suggesting that the intact and functioning host immune system is involved in the periodontal bone loss. In addition, mice deficient in IL-6 and IFN-γ also demonstrated less bone loss suggesting a role for these specific cytokines in the pathogenesis of periodontal bone loss in this model.

The role of anti-inflammatory cytokines has also been investigated in the mouse model. Interleukin 10 (IL-10) is one such anti-inflammatory cytokine that acts by regulating the expression of pro-inflammatory cytokines such as IL-1 and TNF. Mice deficient in this anti-inflammatory cytokine had significantly increased alveolar bone loss compared to controls with functioning IL-10, these differences were magnified with increasing ages of the mice. The implication was that IL-10 prevents over-expression of innate host pro-inflammatory cytokines that are elicited in response to the constant challenge from the commensal oral microbiota. It could be argued that the development of disease in these knockout mice is due to a disruption to the normal balance between the normal oral commensal microbiota and the innate immune status of the periodontal tissues[71].

2.4.4.3. Toll like receptors in periodontal disease

Innate immune responses to microbial challenge are initiated by recognition of specific structures of the invading micro-organisms (e.g. DNA, flagella, fimbriae) called pathogen-associated molecular patterns (PAMPs). Pattern recognition receptors are the host receptors that recognise these specific microbial components from micro-organisms that have co-evolved with the host[94]. Toll-like receptors (TLRs) are a family of these microbial recognition receptors that recognise PAMPs through amino-terminal leucine rich repeats[95]. There are to date 11 known TLRs.

The role of TLRs in the pathogenesis of periodontal disease has been investigated. In one study[96] plaque samples were taken from individuals with periodontal disease and healthy controls. These samples were used to stimulate TLR activity in a cell line of Chinese hamster ovarian reporter cells. It was found that the plaque from individuals with inflammation was significantly stronger stimulators of TLR4 but that all plaque samples could stimulate TLR2 and TLR4 activity. In *P.gingivalis* challenge models of periodontitis TLR2 has been shown to be important in the response to the infecting bacteria[97]. TLR2 is required to mount an immune response to *P. gingivalis* and in the absence of this receptor the pro-inflammatory cytokine levels are reduced and there is resultant decreased periodontal bone loss. Despite this the bacteria is rapidly cleared when compared to wild type mice suggesting TLR2 driven inflammation may hinder the overcoming of the bacterial insult.

It has been shown that *P. gingivalis* interferes with the innate host protective mechanisms by inducing crosstalk between TLR2 and C-X-C-chemokine receptor 4 (CXCR4)[98]. The fimbriae of *P. gingivalis* can induce CXCR4/TLR2 association in lipid rafts that can interact with both receptors. Binding to CXCR4 leads to inhibition of the pro-inflammatory TLR2 mediated pathway and activation of host responses that would be involved in clearing the bacteria, thus this is a means in which *P. gingivalis* evades host mechanisms tasked with its clearance. This is an example of how a pathogen can manipulate the innate host responses to survive.

TLR4 activity has been shown to be influenced by *P. gingivalis* LPS. The lipid A component of LPS can vary in structure existing in different forms that interact with TLR4 in different ways; these include a penta-acyl di-phosphorylated form which is a weak agonist of TLR4 and a tetra-acyl monophosphorylated form which is an antagonist at TLR4[99]. *P. gingivalis* can modulate the expression of its lipid A structure such that the TLR4 antagonist is dominant. Consequently, TLR4 is not activated and the cascade that leads to the expression of pro-inflammatory cytokines is not produced[100, 101]. The modulation of the expression of the lipid A of *P. gingivalis* is dependent on the haemin concentration[102] with the tetra acylated form being favoured by high haemin concentrations. Consequently, the TLR4 antagonistic form of lipid A is expressed when inflammation is present further dampening the pro-inflammatory response and allowing survival of the pathogen.

2.4.4.4. The role of matrix metalloproteinases in periodontal disease

Matrix metalloproteinases (MMPs) are a group of destructive enzymes, which include collagenases, and are responsible for the breakdown of collagen fibres in the gingival tissues. These important host derived enzymes degrade the collagen and extracellular matrix (ECM) of the periodontal tissues. There are around 25 members of the MMP family sub-classified into 6 groups[103]. The activity of the MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs) and it is the ratio of these 2 enzymes that determine whether there is overall collagen destruction i.e. if MMPs>TIMPs.

2.4.4.4.1. Collagenases

The collagenases are the major group of MMPs involved in periodontal tissue breakdown, namely MMPs 1, 8, 13 and 18 and of these MMP-8 has been the most thoroughly investigated. This enzyme has the unique property of the collagenases of being able to break down both type I and type III collagen. MMP-8 is produced by neutrophils and has been shown to be elevated by sixfold in the gingival crevicular fluid (GCF) of individuals with periodontal inflammation[104]. Those individuals with gingivitis had only modest increases and there was no activity in the GCF of healthy controls. Levels of MMP-8 have also been shown to be reduced by periodontal therapy and further reduced by ongoing periodontal maintenance therapy[105]. These findings have been supported by other investigators[106, 107].

MMP-13 is another collagenase and has been shown to induce bone resorption[108]. These investigators found that levels of MMP-13 were increased in periodontal disease active sites and *in vitro* MMP-13 has been shown to activate MMP-9 which may lead to a cascade of MMP activity and ensuing tissue destruction.

2.4.4.4.2. Gelatinases

The gelatinases are enzymes that are able to degrade type IV collagen and include MMP-2 secreted by gingival fibroblasts and MMP-9 secreted by polymorphonuclear leukocytes (PMNLs). Levels of MMP-9 was elevated in the GCF of individuals with periodontal disease compared to gingivitis or healthy controls whereas the converse was true for MMP-2[109]. MMP-9 has been shown to decrease after periodontal therapy[107] and has been shown to be further elevated in smokers with chronic periodontitis before treatment[110].

2.4.5. Conclusions

As can be seen there are a host of complex and interacting regulatory systems within a host that are targeted with controlling the constant insult due to the oral commensal microbiota. This tissue homeostasis is vital in maintaining the health of the host. There are a multitude of bacterial components that can potentially disrupt this homeostasis and tip the balance towards the disease state. Periodontal disease can be considered as a disease caused by the polymicrobial disruption of host homeostasis[71].

2.5. Microbiology of Periodontal Disease

It is widely agreed that periodontal disease is initiated and propagated by aggregations of micro-organisms that colonise the subgingival environment; a plaque biofilm. The micro-organisms then initiate an inflammatory and immune response in a susceptible host that leads to tissue damage.

All mucosal surfaces of the body are routinely colonised by micro-organisms which largely live in harmony with the host. Each surface has a community of micro-organisms, or microbiota, that are uniquely developed to live in that specific ecological niche. The total collection of the microbiota from the mucosal surfaces is termed the human microbiome. The individual ecological niches are in a constant state of balance between the bacteria and the host. It is this symbiotic balance that is vital to the maintenance of health of the host and development of disease.

The oral environment is a unique site in the human body providing a highly specific environmental niche. Mucosal sites elsewhere in the body are constantly shed and replaced which is a primary mechanism for preventing excessive build up of the microbiota and part of the homeostatic mechanisms of the host. In the mouth the situation is different in that there is a non shedding surface, the teeth, which breach the mucosal surface. This has two important effects; firstly, the teeth are non-shedding so the microbiota can accumulate unchecked and secondly, there is a potential discontinuity in the mucosal surface due to the breaching by the teeth (an intact mucosal surface is the primary defence in preventing the ingress of infective micro-organisms). In addition, this ecological niche provides optimal habitat for bacterial growth in terms of stable temperature, water supply and nutrient supply.

The evolutionary development of this niche has occurred in conjunction with the development of host defence mechanisms specialised to this site. These include the washing action of gingival crevicular fluid which includes anti-bacterial agents derived from plasma such as complement as well as a keenly developed and co-ordinated host inflammatory response and innate immunity. The formation of the biofilm itself is influenced in a positive way by the ecological niche such that micro-organisms that are 'protective' are selected over those which may be harmful to health. Indeed, the pathogenesis of periodontal disease can be viewed as a breakdown of homeostasis between the host tissues and the commensal microbiota. This disruption in homeostasis leads to changes in the composition of the microbiota with more pathogenic micro-organisms being selected. Hence the composition of the microbiota is altered in an unfavourable way. This alteration is termed dysbiosis[111], leading to microbial imbalances.

The oral cavity including the subgingival niche is home to over 600 prevalent taxa of different micro-organisms[112] with the majority being uncultured (68%). Despite this, in the majority of individuals this does not lead to periodontal disease. Current evidence indicates that periodontitis occurs in predisposed individuals who have an abnormal inflammatory/immune response to specific organisms within the microbial plaque biofilm that accumulates at the gingival margin[85].

2.5.1. Historical perspective

Discoveries of the association of a bacterial plaque biofilm and specific contituent micro-organisms have largely followed the development of the technology to discover and describe them. From the discovery of 'animacules' by Atonie van Leeuwenhoek in plaque from human teeth under the newly invented microscope in 1675 to more modern non culture sequencing techniques our understanding of the microbiological pathogenesis of periodontal disease has developed.

2.5.2. Microscopy

Early studies in the 20th century using microscopic techniques to examine subgingival plaque samples identified potential aetiological agents[113]. These were as follows

- Streptococci
- Amoeba
- Spirochaetes
- Fusiform bacteria

All of these were found in greater numbers in the plaque samples of individuals with the signs and symptoms of periodontal diseases. However, the evidence was equivocal and not consistently repeated in follow up studies which looked at the effects of treatment on the composition of the plaque biofilm.

2.5.3. Anaerobic culture techniques

Having described the unique ecological niche of the periodontium, the presence of a periodontal pocket provides an environment which becomes increasingly anaerobic as periodontal disease develops and the pocket deepens. Consequently, the next significant breakthrough in discovering a microbiological aetiology for periodontal disease came with the advent of anaerobic culture techniques which offered the opportunity to more reliably culture micro-organisms that were obligately anaerobic.

These studies[114-119] were extremely time consuming and labour intensive and consequently only involved samples of plaque from a few individuals. However, results did begin to show that there were differences in the oral microbiota from individuals with periodontal disease compared to healthy individuals. In addition, specific micro-organisms began to emerge as commonly associated with disease.

Probably the most extensive series of studies using these techniques were carried out at Virginia Polytechnic in the 1980s[120-123]. This group examined the cultivable anaerobic microbiota in different periodontal diseases; gingivitis, juvenile periodontitis and severe chronic periodontitis. Following culture of the micro-organisms under anaerobic conditions, these bacteria were then isolated and identified based on morphological, serological and biochemical methods.

These studies typically involved hundreds of plaque samples from diseased and healthy individuals. In their study looking at moderate chronic periodontitis[121] 1900 bacterial isolates were produced from 60 individuals producing a total of 171 taxa of bacteria. They found significant differences between the composition of the subgingival microflora and the supragingival microflora and from the subgingival microflora of healthy controls. This subgingival microflora was not significantly different from younger adults affected with periodontitis from an earlier study[120]. In a later study[122] this group compared the subgingival microflora of individuals with juvenile periodontitis, which was being considered as a distinct disease entity, with adult forms of the disease. It was found that there were significant differences in the subgingival microflora in individuals suffering from the juvenile forms of the disease with healthy controls or children with (experimental) gingivitis. The subgingival microflora was however similar to adults with (experimental) gingivitis. Of the 18,000 isolates identified, 54 non-treponemal species (e.g. *Fusobacteria sp*, *Bacteroides sp*), 2 treponemal species (e.g. *Treponema denticola*) and mycoplasma were associated with diseased periodontal sulci.

2.5.4. Molecular DNA analysis of bacteria

Whilst techniques to date had allowed the development of an extensive library of the cultivable components of the oral microbiota the major limitation was the labour intensive nature of culture techniques. In addition, there was the suspicion that there may be a multitude of micro-organisms that could not be cultured and so would remain undetected. Any of these micro-organisms could be important in the pathogenesis of periodontal disease.

With developments in understanding the nature of DNA and progress in other fields of microbiology, techniques were introduced that did not rely on culturing the micro-organism as the initial step in identification of the bacteria – non-culture techniques. The most common methodology involved a specific region, the 16S ribosomal DNA gene (16S rDNA) which is present as multiple copies on the bacterial genome (Figure 2.8). These genes code for 16S ribosomal RNA (16S rRNA) which is a component of the 30S (S meaning sedimentary coefficient in Svedberg units) small subunit of all bacterial ribosomes. The sequences of certain regions of the gene (16S rDNA) are highly conserved between different bacteria allowing amplification by Polymerase Chain Reaction (PCR) of all the bacteria present by designing primers to the conserved regions of this gene. In addition, there are hypervariable regions within this gene that vary between different micro-organisms so, by designing primers to amplify the sequences within this variable region, the PCR will only produce amplicons from the pool of target bacteria.

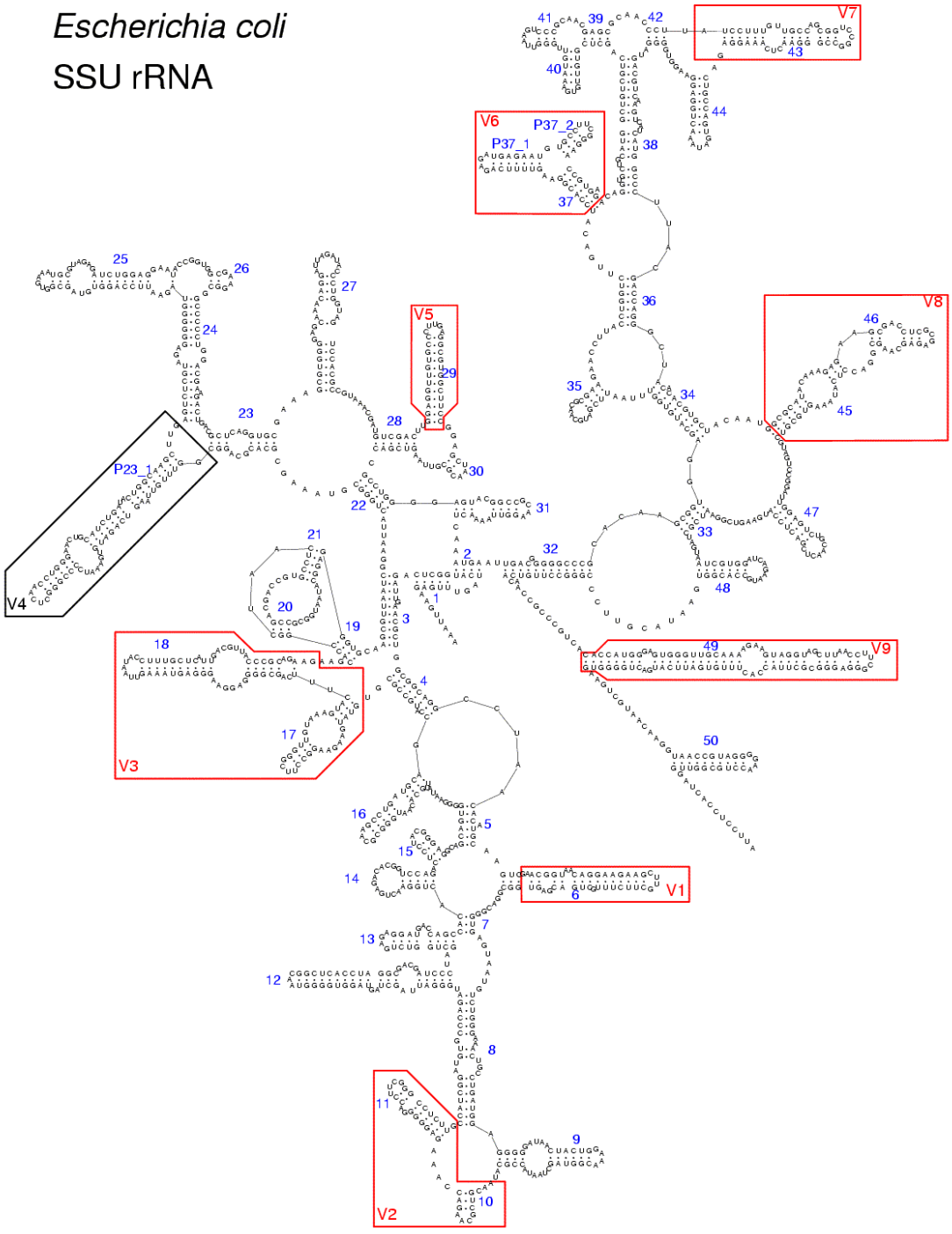


Figure 2.8 The 16S rDNA gene for *Escherichia coli* with the 9 hypervariable regions (V1-V9) shown in relation to their base position along the 16S rDNA gene (from the 5' end). Image courtesy of http://rrna.uia.ac.be/ssu/ [124].

2.5.5. DNA:DNA checkerboard techniques

Using this technique simultaneous hybridisation of up to 45 individual samples from periodontal plaque could be investigated with up to 30 different DNA probes in one assay. The DNA probes are either whole genomic DNA from target bacteria or PCR amplicons of regions of the 16S rRNA gene specific to target bacteria. These probes hybridise to target bacteria and this reaction is linked to a fluorescent signal molecule which fluoresces at an intensity that corresponds to the proportion of target bacteria DNA present.

The pioneering investigation[125] using whole genomic DNA hybridisation analysed 13,261 plaque samples from the mesial aspect of each tooth in 185 individuals with or without periodontitis. Whole genomic DNA probes were used to 40 subgingival taxa and DNA:DNA checkerboard analysis performed. These investigators then used statistical tools to group the various bacteria and investigate their associations with periodontal disease. One major group consisted of 3 bacteria, *Bacteroides forsythus* (now *Tannerella forsythia*), *Porphyromonas gingivalis* and *Treponema denticola* and these were strikingly associated with clinical measures of periodontal disease especially increased probing pocket depth and increased bleeding on probing. In total 5 major complexes were designated and their association with periodontal disease graded and expressed as a traffic light system: red complex (*B. forsythus* (now called *T. forsythia*), *P. gingivalis* and *T. denticola*) being most strongly associated through to the weaker associated orange and yellow complexes and finally to the bacteria associated with periodontal health; the green complex (Figure 2.9).

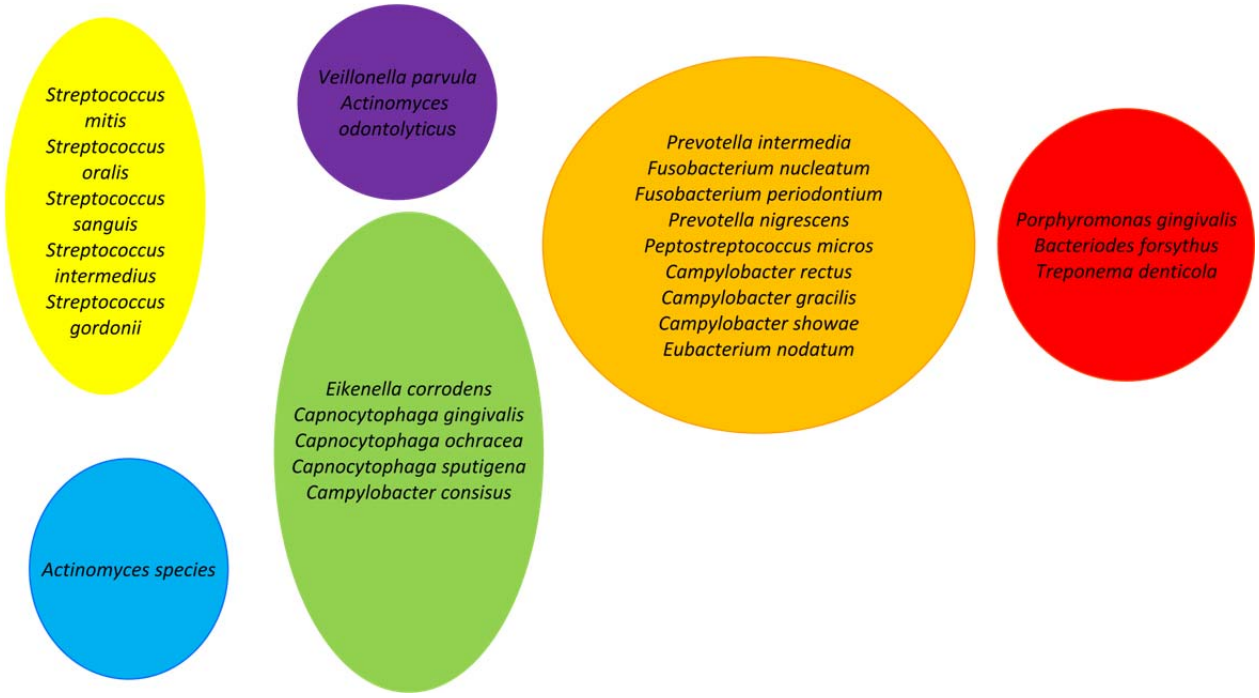


Figure 2.9 The associations of micro-organisms into complexes in subgingival plaque and association with periodontal disease state; the red complex organisms being most strongly associated with periodontal disease[125].

Interestingly, the associations of the different complexes with periodontal disease mirror their colonisation and succession; micro-organisms of the green and yellow complexes being the early colonisers, the orange complex bacteria being later colonisers. These are thought to pave the way for the red complex to colonise much later on and initiate the pathogenesis of the disease.

Despite *Aggregatibacter actinomycetemcomitans* being associated with rapidly progressive disease in a demographic of North and West African descent[126] in this analysis it did not cluster with the red complex organisms. The aggressive periodontal disease in this group of individual is associated with a specific mutation in the *jp2* gene of the bacteria and the influence of host genetic background is a key determinant in the pathogenesis of the disease. In the non-specific host genetic background of the checkerboard analysis the association with disease is much weaker.

In addition this type of analysis permitted site specific analysis to assess the impact of periodontal therapy[127]. In this study samples from sites were assessed at baseline and then 12 months following periodontal therapy. Probes were directed at 40 micro-organisms and it was found that at sites where there was a gain in clinical attachment level following therapy there was a decrease in the numbers of red complex organisms detected.

2.5.6. Sequencing of the 16S rRNA gene

Specific PCR primers designed to anneal to the conserved regions to amplify the hypervariable and bacterial species specific region of the 16S rDNA gene can be used to detect target micro-organisms. This technique is highly sensitive and allows detection of multiple bacteria in the same reaction.

Having identified that certain micro-organisms were strongly associated with periodontal disease these were investigated further using this targeted amplification methodology. The association of *Bacteroides forsythus* (now called *T. forsythia*) and *Porphyromonas gingivalis* with health and disease was explored using this technology[128, 129]. Samples from 291 subjects were analysed and amplification of the 16S and 23S genes performed using universal primers. These amplicons were then further amplified using specific oligonucleotide primers for the 2 target micro-organisms. The results showed that *Bacteroides forsythus* (now called *T. forsythia*) was strongly associated with disease but that another closely related phylotype BU063 was associated with oral health. In addition, the 2 phylotypes of *Bacteroides sp* were found together in the same subgingival sample less often that would be predicted by chance, hence it was concluded that there was some exclusivity mechanism between the 2 *Bacteroides* species. In the earlier study[128] *P. gingivalis* was detected in 25% of healthy subject but 79% of individuals with periodontitis with a resultant odd ratio for being infected with *P. gingivalis* of 11.2 greater in the periodontitis group than the healthy. Colonisation with *P. gingivalis* was independent of the presence of either species of *Bacteroides*.

This technology has also been useful in distinguishing between phylotypes and has also highlighted novel micro-organisms which were previously undetected[130]. In this study these investigators used specific primers for the genera *Porphyromonas* and *Tannerella*. These were used on samples from 5 age and gender-matched pairs, one had periodontitis (2 or more pockets >6mm) and the other was healthy (no pockets >4mm). 308 clones were sequenced and found to belong to 6 *Porphyromonas* or *Tannerella* species or phylotypes, one of which was novel; *Porphyromonas* P3. *Tannerella forsythia* was found in higher proportions in individuals with periodontal disease than in health.

2.5.7. Real time PCR analysis

The type of qualitative PCR technique described above has its limitations in the information supplied i.e. only the positive or negative detection of a specific micro-organism. Developments in real-time polymerase chain reaction (qPCR or qRT-PCR) have allowed quantification of the target micro-organisms along with their detection.

Early studies using this approach used the TaqMan system to determine the amount of *P. gingivalis* ATCC33277 present in plaque samples from individuals with periodontitis and healthy controls. They used universal primers to detect the total number of all bacterial cells present and *P. gingivalis* specific primers to detect and quantify this micro-organism. The percentage of *P.gingivalis* compared to the total bacteria present was significantly higher in individuals with periodontitis (typically 0.00-7.1%) when compared to healthy controls (typically 0.00-0.26%) although this was subject dependent.

Further development of these techniques showed that the sensitivity for detecting *P. gingivalis* in plaque samples was as few as 1 colony forming unit (CFU)[131]. When compared to culture techniques these authors found that *P. gingivalis* was detected in 43% of 259 subgingival plaque samples by culture and in 53% by qPCR.

A similar approach has been used to look at other pathogenic micro-organisms such as *Aggregatibacter* (at that time *Actinobacillus*) *actinomycetemcomitans* and *Prevotella intermedia*[132]. In this study plaque samples were taken from 8 individuals (8 periodontal pockets) at baseline and 1 week following local delivery of minocycline. Unsurprisingly, the authors found that the numbers of these 2 micro-organisms as well as *P. gingivalis* decreased significantly following delivery of the antibiotic. They also looked at levels of *tetQ* gene with specific primers; this gene is implicated in the development of antibiotic resistance to tetracycline. A higher number of copies of this gene were present following antibiotic therapy.

A more recent meta analysis has been performed on the ability of this qPCR methodology to detect *A. actinomycetemcomitans* and *P. gingivalis* in plaque samples from individuals with disease and in health[133]. It was concluded this technique offered a high degree of diagnostic accuracy when compared to culture methodology.

2.5.8. Human Oral Microbiome Project

Probably the most important application of these DNA sequencing technologies is in the development of the Human Oral Microbiome Database (HOMD) which is an attempt to determine all the species of micro-organisms present within the oral microbiota[112]. The aim of this project was to collate all the gene sequences generated by these DNA sequencing techniques into a single database and then to determine the relative abundance of these micro-organisms and attempt to identify novel taxa. 619 taxa in 13 phyla were identified and of the 36,043 gene sequences examined 24% were named, 8% were cultivated but unnamed and 68% were uncultivated.

2.5.9. Next generation DNA sequencing

The latest development in DNA sequencing and its use in detecting micro-organisms is with high throughput sequencing. As the name suggests this allows analysis of many more samples than is possible by conventional Sanger sequencing techniques.

As proof of concept of this technology, one recent study looked at the oral microflora of healthy adults[134]. Saliva and plaque samples were collected from 71 individuals, the DNA extracted and the variable region (V6) of the 16S gene amplified with primers that incorporated adapters for the 454 pyrosequencer. More than 273,000 PCR amplicons were sequenced of which around 197,000 passed the quality control. 99.6% of sequences belonged to 7 major phyla and of these Actinobacteria, Fusobacteria, and Spirochetes were overexpressed in plaque samples while Bacteroides, Firmicutes and Proteobacteria were more prevalent in saliva. At a genus level 185 genera were identified in saliva and 267 in plaque with 11% unidentifiable at this level. In terms of strain, 5600 phylotypes were found in saliva and 10,000 in plaque. The overall conclusion was that 19,000 phylotypes were present in the oral cavity. This is much greater than previously thought by a factor of 10.

The sensitivity of this methodology has been investigated using high throughput sequencing of serial dilutions of saliva (as well as nasopharynx and faecal material)[135]. Once the threshold of $<10^5$ bacteria was reached the sequence profiles produced began to differ from the original undiluted samples e.g. significant increases in Proteobacteria and decreases in Bacteroides. From this study a minimum threshold of DNA concentration was proposed of 1pg μl^{-1}.

In addition, concerns have been raised about inherent errors in this technique especially the issue of homopolymeric tracts, areas in which a single base is repeated a number of times (e.g. CCCCCCC) and the algorithm cannot separate out the exact number present. This can lead to over estimation of the number of unique sequences in a sample. More recent studies have been more conservative in their estimates of the number of components of the human oral microbiome[136]. Using similar 454 pyrosequencing technology to define the microbiota in samples taken from the teeth, cheek, hard palate, tongue and saliva in 3 healthy individuals. 3600 unique sequences with over 50 phylotypes were detected.

A number of next generation sequencing platforms are available commercially and these have been evaluated and compared using samples from an outbreak of *E coli* 0104:H4 in Germany in 2011[137].

2.6. Periodontal disease as an infectious disease

For periodontal disease to be a truly infectious disease any infectious agent must satisfy Koch's postulates[138]

- The micro-organism must be found in abundance in all organisms suffering from the disease and not in healthy organisms
- The micro-organism must be isolated from the diseased organism and grown in pure culture
- The cultured micro-organism should cause disease when introduced into a healthy organism
- The micro-organism must be re-isolated from the inoculated, diseased experimental host organism and identified as being identical to the original specific causative micro-organism

However, it has been shown by modern non-culture techniques that the majority of the oral commensal human microbiome is not cultivable so would not satisfy these criteria.

Socransky[139] modified these criteria to allow determination of a potential periodontal pathogen based upon the weight of evidence rather than absolute fulfilment of Koch's postulates. These criteria were

- Association with disease; a putative pathogen must be found at significantly greater levels at diseased sites compared to healthy sites
- Elimination of the micro-organism; elimination or suppression of the putative pathogen should halt progression of the lesion
- Host response; the host response to a putative pathogen should be elevated compared to the host response to non pathogenic micro-organisms
- Animal pathogenicity; the putative pathogen should be able to colonise the mouths of germ free or conventional animals and cause a disease state similar to that observed in humans
- Mechanisms of pathogenicity; the putative pathogen should possess characteristics that could allow it to contribute to the pathogenesis of the disease

However, there are still limitations in that it is unlikely that periodontal disease is caused by a single micro-organism so this has led to the proposal of a number of micro-organisms being implicated as putative periodontal pathogens[140]. In addition elimination of single components of the plaque biofilm is not possible and so disease remission due to this would not be possible to observe.

In the light of the development of molecular techniques for the identification of the bacterial components of the oral microbiome and especially the identification of the non-cultivable components these postulates have been revised further[141].

- The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species
- Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence
- Reversion or allelic replacement of the mutated gene should lead to restoration of the pathogenicity

This reasoning led to two alternative hypotheses for the relationship between plaque and periodontal disease, the specific and non-specific plaque hypotheses. These ideas have been updated with the concept of the ecological plaque hypothesis. An explanation follows in the next section.

2.7. Plaque as an aetiological agent in periodontal disease

As can be seen from the preceding sections the links between micro-organisms in the form of subgingival plaque and periodontal disease are multiple. This had led to a number of differing hypotheses for plaque as an aetiological driver of periodontal inflammation.

2.7.1. Non-specific plaque hypothesis

The association of periodontal inflammation with a plaque biofilm has been observed for nearly 200 years. The non-specific plaque hypothesis suggests it is the number of micro-organisms accumulating on the root surface which determine the severity and progression of periodontal disease rather than the specific micro-organisms present. This was supported by evidence that the numbers of cultivable micro-organisms increased as disease progressed and subgingival niches deepened but that no single species appeared in active sites that did not appear in healthy sites. It was concluded that many different combinations of indigenous micro-organisms have the pathogenic potential to cause disease[142]. However, this does not explain the full story; there are certain individuals who harbour huge accumulations of dental plaque but exhibit no signs of periodontitis. Moreover, there is overwhelming evidence that there are significant differences in the composition of dental plaque between health and disease. These observations led to the specific plaque hypothesis.

2.7.2. Specific plaque hypothesis

This was proposed in the 1970s and suggested that only a small number (around twenty) of the hundreds of microbial species present in plaque are causative of disease[143]. Moreover, their numbers increase in the development and progression of the disease. This hypothesis is supported by some of the conclusions of the 1996 World Workshop[140]. Later DNA:DNA hybridisation studies[125] showed that co-aggregations of certain complexes of specific micro-organisms were strongly associated with periodontal inflammation.

2.7.3. Ecological plaque hypothesis

In this hypothesis it is the micro-environment of the niche in which the dental plaque resides that is the key driver of the nature of the biofilm present and the degree of periodontal inflammation. Variations in conditions (e.g. pH) select certain micro-organisms that are optimised to thrive in these conditions over others that are less well adapted. Thus the balance in composition of the biofilm is established. In the disease state the environmental conditions are different e.g. altered inflammatory exudates containing different nutrients and host defence strategies.Consequently, the balance in composition of the microbial community changes.

2.8. Biofilms

Biofilms can be defined as 'matrix enclosed bacterial accretions adherent to each other and/or surfaces or interfaces'[144]. They have been discovered in the fossil record dating back 3.25 million years and appear associated with a diverse range of organisms. Their formation is an ancient and integral component of prokaryotic life and confers the ability to survive in a diverse range of different environments[145].

Biofilms offer a microniche for the bacteria to survive and resist varying degrees of challenge. Biofilms bacteria are at least 500 times resistant to antibacterial agents[146]. The cells comprising a biofilm are phenotypically different from their counterparts in planktonic growth. In addition, there are more cells within a biofilm than in planktonic growth and these cells are fixed in position by the extracellular matrix relative to other micro-organisms allowing mutual co-operation and symbiosis[144].

The oral environment is somewhat different from other sites in the body in that the tooth is a non shedding surface which means that large quantities of micro-organisms can accrue if left unchecked. However, these accumulations of micro-organisms display properties that are typical of biofilms[147]. These include communication by gene transfer and functional organisation for increased metabolic efficiency, greater resistance to stress and ultimately enhanced virulence.

This mode of existence confers a number of advantages over single-cell or planktonic growth[148];

- Colonising species are protected against competing micro-organisms
- Species are protected from the host defences
- Species are protected from potentially toxic environmental substances
- Nutrient uptake is facilitated
- Cross-feeding between bacterial species
- Removal from potentially harmful metabolic products
- Provision of appropriate physiochemical environment (e.g. pH, oxygen tension)

2.8.1. Biofilm structure

2.8.1.1. Biofilm superstructure and extracellular matrix

Plaque biofilms are highly ordered eco-systems of various colonies of cells (15-20% by volume) that are arranged in co-aggregations and non-randomly distributed in a glycoclayx matrix[148]; an extracellular polymeric structure of glycoproteins, proteoglycans and glycolipids. This exopolysaccharide matrix is produced and maintained by the developing biofilm and confers a number of advantages to the community. The component fibres of the matrix are heterogeneous with variations in size, composition and rigidity which are able to interact with each other and with the enclosed micro-organisms. This superstructure is also protective of its enclosed cells by preventing excessive moisture loss and also acting as a store of nutrients. It also protects against the ingress of potentially damaging components of the host inflammatory and innate immune responses.

In situ confocal laser scanning of dental plaque biofilms has shown that these eco-systems are highly organised[149]. There are water filled voids and channels within the superstructure which may act as a primitive circulatory systems[150]. There is also variation in the overall shape of the biofilm which is dependent on the environment in which it grows. The main determinant of shape are the shear forces in the fluid medium in which the biofilm is growing. Lower shear forces are associated with mushroom shaped structures[151] whilst greater shear forces (greater fluid flow) tend to produce more elongated structures that are capable of more rapid oscillation[152] and a more dynamic biofilm.

2.8.2. Biofilm formation and bacterial succession

The primary step in the formation of any biofilm is the attachment of bacteria to a surface. In supragingival areas, salivary glycoproteins and antibodies adsorb on the clean surface of a tooth to form the acquired pellicle[153]. This pellicle provides a means of attachment for a number of oral bacteria to bind. Using DNA:DNA checkerboard analysis these early colonisers were identified and characterised[154]. The developing biofilm in 15 individuals was monitored at baseline, 2, 4 and 6 hours after cleaning. This was also compared to whole saliva. The results showed that there was selectivity in the adhesion process with the composition of the developing biofilm differing from the bacteria in the saliva. On the tooth surface *Actinomyces sp* were the initial colonisers but with time the biofilm began to diversify and *Streptococcus sp* (mainly *S. mitis* and *S. oralis*) began to be introduced. The data showed periodontal pathogens such as *Tannerella forsythia*, *Treponema denticola*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were only present in very low levels in this initial phase of biofilm development in this healthy group of subjects.

This has also been examined by more conventional 16S rRNA sequencing techniques[155]. Retrievable enamel chips were placed in the mouths of 3 healthy individuals and examination of 16S rRNA amplicons at 4 and 8 hours after placement. Despite differences between the subjects this study confirmed that 4 and 8 hour communities were dominated by *Streptococci sp* (*S. mitis* and *S oralis*) with other significant biofilm components being *Actinomyces sp*, *Gemella sp*, *Granulicatella sp*, *Neisseria sp*, *Prevotella sp*, *Rothia sp* and *Veillonella sp*. Interestingly, this study showed that Gram-negative species such as *Prevotella sp* and *Porphyromonas sp* were sometimes present confirming they can be early colonisers.

These findings are reproducible and seem to suggest that despite individual variation that the initial biofilm is largely made up of *Streptoccocus sp*. This situation is relatively stable until around 2 days[156] at this time the plaque biofilm matures and changes. Changes are brought about by alterations in the local environment as the biomass accumulates allowing minor components of these intial communities to thrive and predominate[157].

After 7 days of plaque accumulation the nature of the microbiota shifts to more filaments and rods[156] with the introduction of spirochetes[158]. Studies have used DNA:DNA checkerboard analysis to look at the developing and maturing plaque biofilm[159]. Ten healthy subjects abstained from oral hygiene measures for 4 days and had samples taken of the developing biofilm. The checkerboard analysis showed that following early colonisation with *Actinomyces sp* and *Streptococcus sp* as the biofilm matured orange complex micro-organisms began to significantly increase with time (e.g. *Campylobacter sp* and *Fusobacterium sp*).

Analysis of mature plaque using 16S rRNA gene sequencing has also been performed[160] especially comparing the nature of the subgingival plaque in biofilms from individuals with various forms of periodontal disease and in health. The most striking feature is the highly diverse and complex nature of a mature plaque biofilm consisting of 347 species or phylotypes in 9 bacterial phyla.

Co-aggregation of specific bacterial species is an important property of the development of the plaque biofilm. One such co-aggregation between *Fusobacterium nucleatum* and *Porphyromonas gingivalis* has been known about for nearly 40 years following electron microscope studies in the 1970s[161, 162]. These 2 micro-organisms form 'corn cob' structures in maturing biofilms and their formation is dependent on the partner cell shapes and relative ratios; when the *Porphyromonads* reach a 10 fold excess over the filamentous *Fusobacterium sp* the co-aggregation results[157]. These types of co-aggregation are also possible with other species e.g. *Actinobacillus sp*, *Veillonella sp* and *Streptococcus sp*.

Formation of plaque biofilms is facilitated by structural components of the bacteria. Many micro-organisms have surface fimbriae or fibrils that aid this attachment e.g. *Porphyromonas gingivalis*[163]. The pellicle itself consists of a variety of sites which act as receptors that are recognised by bacteria, mainly *Streptococcus sp*[157]. There are also specific adhesion-receptor interactions between different bacterial species that facilitate co-aggregation and development of a mature plaque biofilm (Figure 2.10).

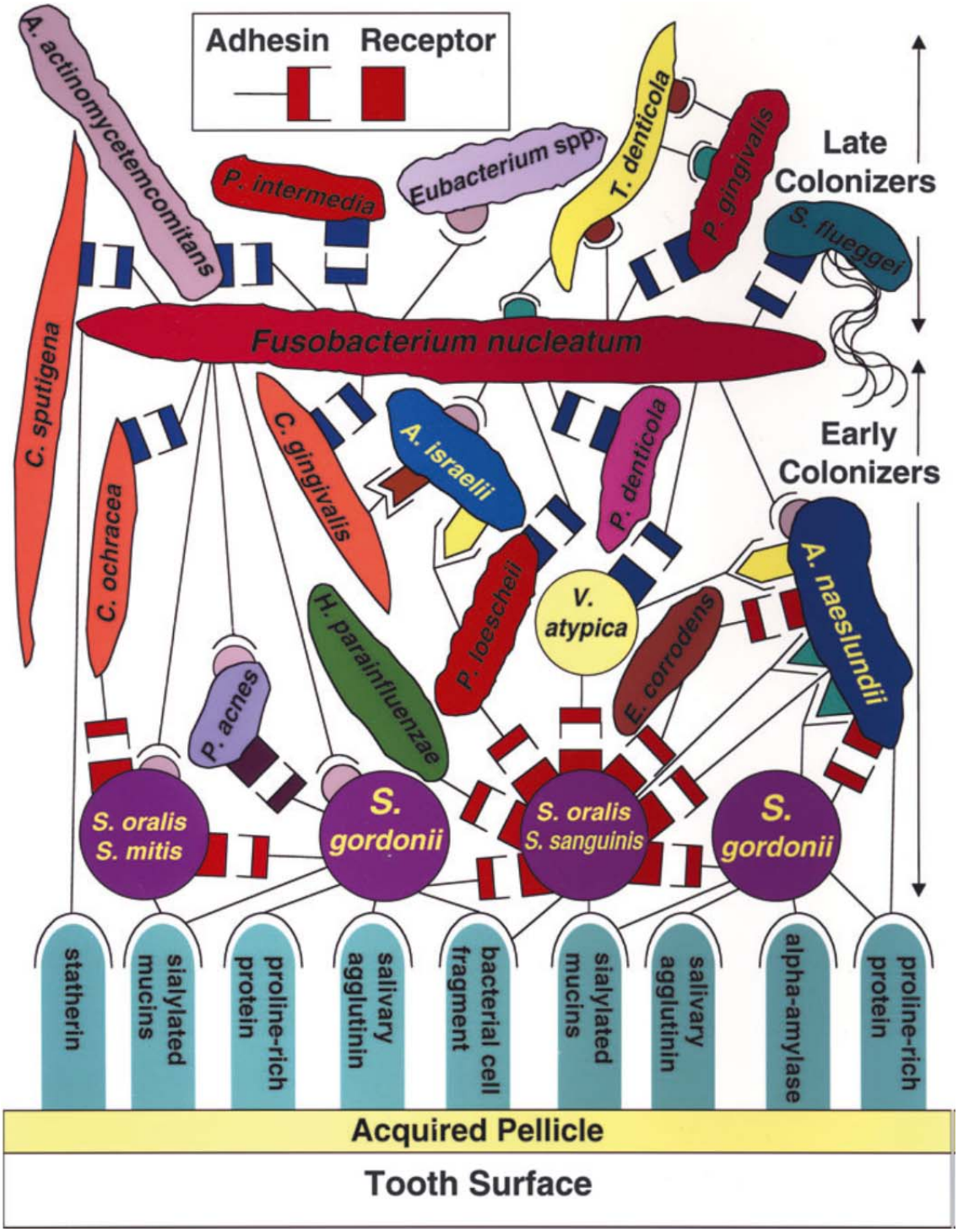


Figure 2.10. The development of the plaque biofilm, from early colonisation to maturity, showing the various cell-cell and cell-surface interactions that facilitate its formation[157].

2.8.3. Genetic variation of micro-organisms within a plaque biofilm

The environment within a biofilm is considerably different from that of free living micro-organisms and this can significantly affect the properties of the micro-organisms. Bacteria within a biofilm can display a different phenotype from the same cells living in planktonic environment[164].

In its simplest form the process of adhesion of bacterial cells to a surface and the resultant biofilm lifestyle can lead to alterations in the gene expression of the cells. *Streptococcus mutans* was allowed to adhere to a glass slide for 2 hours and then the expression of proteins was compared to the same micro-organism in planktonic medium[165]. The synthesis of 25 proteins was enhanced and 8 proteins diminished by the adherence to the glass slide. Most of the changes in the profile of the expressed proteins were a relative increase in those involved in carbohydrate catabolism i.e. a shift towards energy production. In addition biosynthetic proteins had been found to be expressed to a greater degree[166].

Modification of gene expression due to these different growth conditions has been shown in *Porphyromonas gingivalis*[167]. There was found to be an 18% difference in the genes expressed in the micro-organisms when grown in a biofilm (artificial flow cell) compared to planktonic growth. There was down regulation in the biofilm micro-organisms of genes responsible for DNA replication, cell envelope biosynthesis, energy production and biosynthesis of cofactors. There was a concurrent up regulation in a number of genes that encoded signal transduction and transcriptional regulation. It might be argued that there was a down regulation in the genes required for survival due to the mutual benefit conferred by the biofilm environment and an up regulation in the genes responsible for signalling with other micro-organisms within the biofilm.

Studies have analysed the total proteome of *P. gingivalis* when grown under planktonic and biofilm conditions[168]. These authors found that 24 proteins increased in abundance and 18 decreased in abundance when the micro-organism was grown in the biofilm state. The increase was in the proteins RgpA, HagA, CPG70, PG99 and transport proteins HmuY and IhtB, metabolic enzymes FrdAB and immunologic proteins.

Investigations have also been carried out into how interactions between specific micro-organisms can alter gene expression. In one study the co-aggregation of *P. gingivalis* as a community with *F. nucleatum* and *S. gordonii* was examined and the different proteomic expression for *P. gingivalis* compared to planktonic growth. This group found 403 proteins were down regulated and 89 proteins upregulated when *P. gingivalis* was part of this co-aggregation. The decreases were in proteins involved in cell shape and envelope formation, biosynthesis (of thiamine, cobalamin and pyrimidine) and DNA repair. The increases were in proteins responsible for protein synthesis and HmuR (a TonB dependent outer membrane haemin receptor). Similar studies have looked at proteomic expression when *P. gingivalis* was grown in a flow cell with the another red complex micro-organism; *T. denticola* [169]. The authors found a change in protein expression that suggested altered iron acquisition by *P. gingivalis* due to large increases in biofilm expressed HusA and HusB (outer membrane proteins involved in iron uptake) whilst other iron/haem transport systems decreased (e.g. HmuY). There were also significant increases in enzymes involved in glutamate and glycine catabolism.

2.8.4. Bacterial interactions within a biofilm

Some functions within the biofilm are dependent on the ability of the micro-organisms to effectively communicate with each other. This allows interaction and collaboration (or inhibition) to confer a survival advantage over certain other bacteria and formation of micro-colonies.

2.8.4.1. Proximity of micro-organisms within the biofilm

Interaction between microbial species within a biofilm can occur by the proximity of them to each other and the summation of their individual effects on the micro-environment around them. *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are obligate anaerobes but can survive in aerobic conditions if they co-aggregate with other oxygen consuming species e.g. *Neisseria sp*, *Actinomyces sp* or *Streptococcus sp*[170]. Antagonistic relationships can also exist between competing biofilm species e.g. the production of bacteriocins by *S.mutans*[171].

2.8.4.2. Quorum signalling within a biofilm

Specific bacterial interactions and communications can occur through small diffusible molecules in a cell density-dependent manner; quorum sensing[172]. Studies using *S. mutans* had shown that the cell density modulated the acid adaptation[173]; higher density of cells were more able to survive the potentially harmful effects of low pH. This effect was found to be dependent on specific genes that coded for a quorum sensing system and that mutations in these genes, leading to defective quorum sensing, resulted in reduced acid tolerance. This quorum sensing system was also found to play an important role in biofilm formation; *S mutans* mutants with defects in the genes (*com*C, *com*D, *com*E, *com*X and *com*CDE) that code for the quorum sensing peptides produced biofilms with lower biomass and altered architecture.

Auto-inducer 2 (AI-2) is encoded by the *Lux*S gene and has been detected in a number of oral Gram-negative and Gram-positive micro-organisms. For example, *A. actinomycetemcomitans* has been shown to rely on *Lux*S-dependent signalling to regulate expression of leukotoxin and iron acquisition[174]. In *P. gingivalis* this *Lux*S-dependent quorum sensing has been investigated in relation to the regulation of Arg-gingipain (Rgp) and Lys-gingipain (Kgp), which are associated with high bacterial cell densities[175]. It was found that this quorum sensing system played a role in modulating protease activity with the mutant strain producing around 45% less Rgp and 30% less Kgp than wild type *P. gingivalis*. In addition, haemagglutination was reduced by fourfold in the mutant strain. Overall, these effects did not affect the virulence of the mutant *P.gingivalis* in the murine lesion model.

2.8.4.3. Gene transfer within a biofilm

The horizontal transfer of genes between micro-organisms within a biofilm is another method of communication. This has been shown as the route by which some antibiotic resistances can be conferred, for example tetracycline resistance in streptococci in model biofilms[176]. The presence of 'pathogenicity islands' in *P.gingivalis* has also been cited as evidence for horizontal gene transfer[177].

It is worth remembering that the biofilm and host have a dynamic relationship that can be altered by many sudden imbalances e.g. the administration of antibiotics. That said a relatively stable microbiota is a key component of the host defences: colonisation resistance[164]. However, this homeostasis can breakdown if there are sudden changes that disrupt this normal balance and favour the selection of previously more minor components of the microbiota. This imbalance or unfavourable alteration in the composition of the microbiota is termed dysbiosis[111].

2.9. Dysbiosis

Dysbiosis is defined as an unfavourable shift in the relative abundancies of individual components of the microbiota in the disease state when compared to health. Technological advances in non-culture techniques have allowed this microbial imbalance in a biofilm to be explored[178]. It is emerging as an important factor in the development of a number of diseases in other organ systems.

2.9.1. Role of dysbiosis in antibiotic associated diarrhea

This is especially true in relation to the gastro-intestinal system. Unsurprisingly, some of the early evidence came from case reports of individuals suffering from diarrhoea associated with antibiotic use[179]. In one such report the administration of Amoxicillin-clavulanic acid in a 39 year old male for acute sinusitis resulted in antibiotic-associated diarrhoea. Detailed analysis of the gut microbiota by 16S rRNA gene sequencing showed distinct changes in the gut microbiota. These changes were reversed following discontinuation of the antibiotic and resulted in resolution of the diarrhoea. This was further examined in patients with the more serious *Clostridium difficile*-associated diarrhoea (CDAD)[180]. Using a 16S rRNA cloning and sequencing approach the gut microbiota of 7 individuals with CDAD was compared with 3 healthy controls. In individuals with CDAD there was a compositional shift in the gut microbiota to a less diverse community.

2.9.2. Role of dysbiosis in inflammatory diseases of the gut

Dysbiosis of the gut microbiome has also been investigated in Inflammatory Bowel Disease[181-183]. Disruption of the delicate balance between the host and its intestinal microbiome results in changes to the mucosal inflammatory response leading to varying degrees of gut inflammation[184]. Comparison of individuals with IBD and healthy controls has been performed using a 16S rRNA sequencing approach[185]. The results from this study showed statistically different gut microbiota between the 2 groups; the healthy controls having greater variation in this microbiome. This dsybiotic microbiome has been associated with changes in metabolic output[186]. The specific change in the gut microbiota in individuals with IBD has been further elucidated[187]. In individuals with IBD there were significant differences in the genera *Coprococcus sp*, *Colinsella sp* and *Coprobacillus sp*.

Gut dysbiosis has also been found in other inflammatory disorders of the bowel. In children with coeliac disease investigators have examined the robustness of the IgA response to the gut microflora, the first line of the innate response to neutralise pathogenic micro-organisms[188]. IgA-coated bacteria in faecal samples were significantly lower in individuals with the disease compared to healthy controls. In addition, the Gram-positive to Gram-negative bacterial ratio was significantly lower in the disease samples. Twin studies have further shed light on dysbiosis in the aetiology of Crohn's disease[189, 190] with specific micro-organisms implicated in the disease namely *Faecalibacterium prausnitzii* and *Escherichia coli* which were present in greater numbers and indicative of an ileal Crohn's disease phenotype.

2.9.3. Role of dysbiosis in colorectal cancer

Dysbiosis of the gut microbiota has also been suggested in the aetiology of colorectal cancer[191]. The stool bacterial DNA of 60 individuals with colorectal cancer was compared with that of 119 healthy controls[192]. Using a pyrosequencing methodology to analyse the microbiome the authors found that the phylogenetic core was different in the cancer patients compared to healthy controls. *Bacteroides sp* and *Prevotella sp* were significantly higher in the cancer group. Other groups have compared the gut microbiome in individuals with colorectal cancer or adenomatous polyposis[193] using 16S rRNA sequencing approaches. There were significant reductions in the temporal stability and increased diversity in individuals with cancer or polyposis; specifically *Clostridium leptum* and *Clostridium coccoides*.

2.9.4. Role of dysbiosis in obesity

There has been recent interest in the links between dysbiosis of the gut microbiome and obesity. The initial reports showed that there were decreases in the proportions of the 'beneficial' group of bacteria *Bacteroidetes* in obese people compared to normal weight controls. This was reversed with weight loss and calorie restricted diets[194]. Twin studies compared obese and lean twins and found that there were phylum levels changes in the gut microbiota between the 2 groups[195] with less bacterial diversity in the obese. This dysbiosis has also been examined in obese individuals before and after gastric bypass surgery[196]. Using pyrosequencing technology 3 normal weight, 3 morbidly obese and 3 individuals post gastric bypass surgery had samples from the gut microbiota examined. In healthy and obese individuals *Firmicutes* predominated but were significantly reduced in the surgery group, the latter having increases in *Gammaproteobacteria*. These changes may be due to alterations to the gut caused by the surgical procedure. The evidence for the links between gut dysbiosis and obesity has recently been reviewed[197] and is an ongoing area of research.

2.9.5. Role of dysbiosis in periodontal disease

The evidence that periodontal disease is the result of a dysbiotic microbiota is evident from the many microbiological analyses of periodontal plaque. We have shown that *P. gingivalis* contributes to the pathogenesis of periodontal disease by bringing about perturbations in the oral commensal microbiota[198]. In these studies the oral commensal microbiota is ultimately responsible for the tissue damage following alterations brought about by the presence, in albeit low numbers, of *P. gingivalis*. This is an area that will be addressed in more detail in the course of this thesis.

2.10. *Porphyromonas gingivalis*, periodontal disease and virulence

The association of *Porphyromonas gingivalis* with periodontal disease has been extensively explored in chapter 2 but has been concisely summarised in a recent mini review[199]. *P. gingivalis* is

- highly associated with chronic periodontitis
- present in up to 85% of disease sites and rarely in healthy sites
- positively correlated with increasing pocket depth
- reduced following treatment and associated with resolution of disease
- implicated in animal models in inducing inflammatory and immune responses

2.10.1. Structure and growth of *P. gingivalis*

Porphyromonas gingivalis is a black pigmenting, asaccahrolytic, non-motile, Gram-negative, anaerobic rod shaped micro-organism (Figure 2.11).

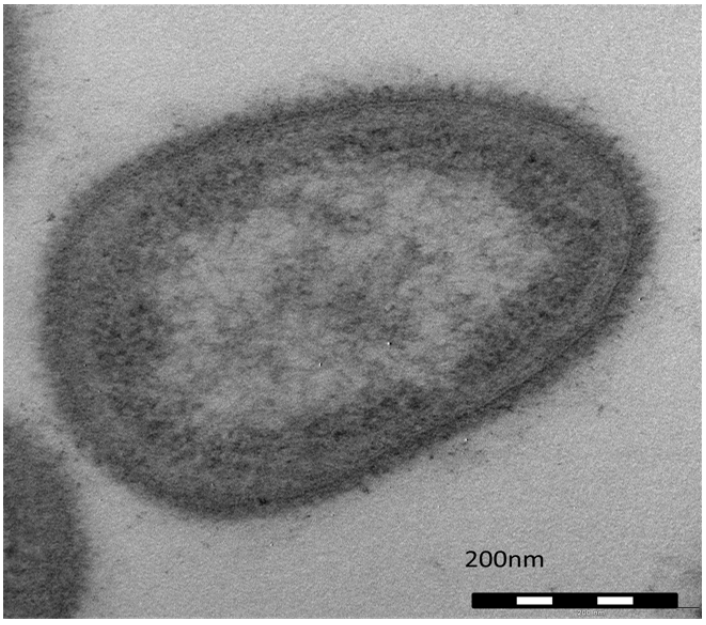


Figure 2.11 Transmission electron microscopy (TEM) of *Porphyromonas gingivalis* W50 at 150,000x magnification. The bacterial cells were fixed in the suspension media with a mixture of glutaraldehyde and formaldehyde. Image courtesy of Dr J Aduse-Opoku.

It is an obligate anaerobe and growth is augmented by the presence of haemin or vitamin K as additional co-factors. The formation of black pigmentation on blood agar is a function of accumulation of μ-oxo bishaemin complexes of iron (III) protoporphyrin IX on the bacterial cell surface[200, 201]. The micro-organism is asaccharolytic in its metabolism which aids its survival in deep periodontal pockets where diffusion of sugars is limited. Energy needs are met by fermenting amino acids and it is able to thrive in iron-limited conditions[202].

P.gingivalis is a late coloniser of the dental plaque biofilm as discussed in section 2.8.2. and is often co-aggregated with other micro-organisms in 'corn cob' arrangements.

2.10.2. Survival strategies of *P. gingivalis*

In order to survive and flourish micro-organisms must be able to evade the host inflammatory and immune response.

2.10.2.1. *P. gingivalis* invasion of host cells

P.gingivalis has been shown to be able to invade oral epithelial cell lines avoiding immune surveillance[203, 204]. In addition, this micro-organism has been found to invade gingival pocket epithelium in surgically excised tissue samples[205]. Studies have also shown that *P. gingivalis* can invade and persist in macrophages[206]. This cellular invasion is mediated by the major fimbriae which bind to host cell surface integrins[207].

2.10.2.2. Subversion of the host defences by *P. gingivalis*

Porphyromonas gingivalis is not a potent stimulator of inflammation[208] but rather subverts the host defence mechanisms through cross-talk with the host[199]. The micro-organism is able to down regulate innate immunity[209] through degradation of host defensins[210], resistance to the oxidative burst of polymorphonuclear neutrophils[211], and inhibition of pro-inflammatory cytokines[212, 213].

2.10.3. Virulence determinants of *P. gingivalis*

P. gingivalis has a number of virulence factors that it can utilise to facilitate its own survival. Virulence factors are molecules that can elicit deleterious effects on host cells[199].

2.10.3.1. Lipopolysaccharide of *P. gingivalis*

A major component of the structure of *P. gingivalis*, and all Gram-negative bacteria, is the lipopolysaccharide (LPS) in the outer membrane. Pattern recognition receptors in the host tissues have an affinity for LPS which enable the host to distinguish between commensal and pathogenic bacteria.

LPS is a powerful inflammatory mediator. In the calvarial mouse model, *P. gingivalis* is a potent stimulator of bone resorption[214]. This was a dose dependent effect and was mediated through TNFα and IL-1. In other animals models, LPS injected directly into the gingiva increased the expression of IL-1α and IL-1β and was associated with histological and histomorphometric signs of bone resorption[215]. *P. gingivalis* is a powerful stimulator of bone resorption in this model but interestingly not as potent or sustained as *A. actimomycetemcomitans*.

In vitro, P. gingivalis LPS is a moderate stimulator of a range of pro-inflammatory cytokines in different cell lines. In one such study[212] blood monocytes were treated with culture supernatants from various bacterial species including *P. gingivalis*. IL-1α levels were induced by all the bacteria tested and interestingly the effect of *P.gingivalis* was the weakest. In addition, during co-stimulation of monocytes with other micro-organisms (*C. rectus*) *P. gingivalis* had an antagonistic effect of IL-1α production. This was concluded to be an important virulence determinant as mixed infection with bacteria that include *P.gingivalis* may lead to antagonistic reductions in IL-1α levels, this cytokine being important in the initial inflammatory response of the host to the infection. Hence the host's ability to mount an inflammatory defence maybe impaired.

The structure of bacterial LPS comprises a conserved lipid A component, a relatively conserved and variable O-antigen which can confer differences in structure and ultimately phenotypic differences between strains. In *P. gingivalis*, the lipid A component can be tetra-acylated or penta-acylated dependent on the local environmental conditions[216] especially the haemin concentration; high haemin availibilty (e.g. in inflammation) the tetra-acylated form is favoured. These two forms appear to have opposing effects on TLR4 receptors and therefore host inflammatory response; the penta-acylated form is an agonist for TLR4 whereas the tetra-acylated form is antagonistic[217]. So in inflammation, where haemin concentration is increased, the tetra-acylated antagonist is favoured and further stimulation of the protective inflammatory response through TLR4 reduced. This adaptability of *P.gingivalis* into two opposing forms (in terms of inflammatory stimuli) allows it to survive in the host.

2.10.3.2. Capsule polysaccharide of *P. gingivalis*

Another structural component of *P. gingivalis* that impacts upon its virulence is the presence of a cell surface polysaccharide capsule. Capsule polysaccharide (CPS) or K-antigen consists of glucose, glucosamine, galactosamine and galactosaminuronic acid[218] and is a stimulator of systemic IgG responses. Based on these responses 6 different serotypes have been distinguished.[219]

Comparisons of the virulence of the 6 capsular serotypes with non capsular strains has been investigated in the mouse model[220]. Virulence was assessed by abscess formation and degree of spreading infection. All strains, capsulated and non-capsulated produced localised abscess but the degree of systemic illness was much more severe for encapsulated forms. In the mice infected with non-capsulated forms of *P. gingivalis* the effects were less severe and the recovery quicker. This has been further investigated by use of a CPS vaccine in the mouse model[221]. When challenged with *P.gingivalis* in the form of oral gavage, mice that had been vaccinated with purified CPS and shown to raise an immune response exhibited lower levels of bone loss.

In vitro investigations have shown that CPS can interact with different host cell lines. In one study the interaction of a human polymorphonuclear leukocytes (PMNs) were tested against 17 strains of *P. gingivalis*. Non-capsulated strains were more readily phagocytosed, the capsule offering a degree of resistance to phagocytosis. This has been further investigated using murine macrophages and challenging with heat killed whole cells and the purified capsular products of the 7 serotypes[222]. Whole CPS and K1 serotype were more stimulatory of pro-inflammatory cytokines than the serotypes K2-K7.

As with LPS, this virulence factor has been shown to be antagonistic to the host inflammatory response, down-regulating the effects allowing evasion and survival[223]. In this investigation a mutant of *P.gingivalis* was created which was unable to synthesis K1 capsule serotype. This mutant was found to be a much more potent stimulator of pro-inflammatory cytokines than the wild type. The inference being that the intact and functional capsule allows a degree of evasion of the host innate immune response.

2.10.3.3. The fimbriae of *P. gingivalis*

Fimbriae are cell surface projections that form thin filaments on *P. gingivalis* that measure 0.3 – 3.0 μm in length and are responsible for facilitating adherence of the bacterial cells to different host substrate molecules and other bacteria. The genetics of this virulence factor was first described in the late 1980s[224].These play an important role in the colonisation of *P. gingivalis* and its inclusion in a developing biofilm. There are 2 types of fimbriae; the major fimbriae (type I) which are mainly involved in colonisation and invasion and minor fimbriae (type II) which are more involved with the stimulation of the host inflammatory response[225]. Based on genetic diversity of the *fimA* gene the major fimbriae have been subclassified into 6 types (Ia, Ib through to V) and certain subtypes of the fimbriae are more strongly associated with individuals with periodontal disease (types II and IV)[226]. The minor fimbriae have been shown to be potent stimulators of pro-inflammatory cytokines IL-1α and β, IL-6 and TNF-α[226].

In vitro in various cell lines the fimbriae have been shown to play an important role in cell invasion. In one study in a cell line derived from monocyte derived dendritic cells, fimbriated strains of *P. gingivalis* 381 were much more effective at cellular invasion that non-fimbriated mutants[227]. This invasion was able to induce the upregulated expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-10, IL-12 and TNF-α.

In animal immunisation experiments using the fimbriae as vaccines, there is evidence that it may offer protection from periodontal breakdown. One group used *S.gordonii* (a human commensal micro-organism and member of the non-periodontitis associated yellow complex) as a vector to express *P. gingivalis* fimbrial components on the cell surface[228]. This vector was immunised into the oral cavity and the immune response monitored through antibody assays (IgG response). Following further oral challenge with wild type *P. gingivalis*, animals that were immunised with the fimbriae were protected from alveolar bone loss and a robust FimA-specific immune response could be demonstrated.

The mechanisms by which the fimbriae induce signalling in host cells has been investigated and 2 distinct receptors seem to be involved; TLR2 and TLR4[229]. Once TLR2 is activated 2 patterns of signalling cascades have been observed; one acting through expression of the pro-inflammatory cytokines IL-6 and TNF-α and the other through cell adhesion molecules such as ICAM-1[230]. Signalling through TLR4 requires concurrent stimulation of membrane protein CD-14 and its activating protein MD-2[231].

2.10.3.4. The gingipains of *P. gingivalis*

On the cell surface of *P. gingivalis* surface cysteine proteinases have been discovered and these can be secreted into the milieu where they have virulence potential. These gingipains have 2 distinct types based on their specificity for a particular substrate, arginine-specific (Arg-X) and lysine-specific (Lys-X)[232, 233]. Arg-X gingipains are sub classified into 2 types; RgpA and RgpB. Both contain a proteolytic domain and in addition RgpA has an adhesion domain. Lys-X gingipain, Kgp,exists in a single form with both proteolytic and adhesion domains[233].

Their virulence potential has been investigated in a number of cell lines. Using a flow cytometric analysis approach human T cells were stimulated with supernatant of *P. gingivalis*[234]. The proteolytic components of both gingipains was able to cleave the T cell surface receptors CD4 and CD8 interfering with cell-mediated immune response. In neutrophil cell lines the proteinases in both gingipains are able to cleave cell surface receptors that play a role in the cell mediated immune response[235]. In gingival epithelial cell lines, RgpB was able stimulate protease activated receptors (PARs) which resulted in intracellular calcium transport and ultimately up-regulation of expression of the pro-inflammatory cytokine IL-6[236]. This activation of PARs by the gingipains has also been investigated in human gingival fibroblasts and T cells[237]. This study assessed the effects of *P. gingivalis* on expression of the gene that coded for a specific receptor (PAR 2) which was found to be up regulated after stimulation with *P. gingivalis* in both cell lines. This up regulation was abolished by heat deactivation of the gingipains. Stimulation of PAR 2 is associated with increases in pro-inflammatory cytokines IL-1α, IL-6, IL-8 and TNF-α[238, 239]. Interestingly, the gingipains have also been shown to proteolytically inactivate certain pro-inflammatory cytokines e.g. IL-12[240, 241] as well as certain anti-inflammatory cytokines e.g. IL-4 and IL-5[242].

The gingipains have potential virulence by interacting with the host complement system. Arg-X gingipain can cleave complement components C3 and C5 in a stepwise and time dependent manner. The degradation of C5 means a potent chemotactic stimulant of neutrophils (C5a) is reduced and hence migration of neutrophils impaired[243]. In addition, the reduction of C3 potentially reduces capacity for opsonisation[244].These gingipains have also been shown to stimulate the release of thrombin from prothrombin, clotting at periodontally involved sites leading to the further release of pro-inflammatory cytokines such as IL-1[245]. Lys-X has been shown to inactivate the C5a receptor on neutrophils hampering their recruitment[246, 247].

Gingipains can have effects on the vasculature of the periodontal tissues. Acting through the clotting cascade they have been shown to proteolytically activate plasma kallikrein and bradykinin as well as prothrombin and thrombin. This leads to increased vascular permeability and influx of neutrophils[248, 249]. In addition, the degradation of fibrinogen has been shown which potentially means reduced coagulation and increased bleeding at infected sites[250] resulting in conditions that favour the growth of *P. gingivalis* in a haemin-rich environment.

The effects of the gingipains would seem to be somewhat contradictory so their exact role as virulence factors is open to interpretation. These variations may be explained by the fact that the release of the gingipains is such that there exists a concentration gradient of these proteinases throughout the gingival tissues[251]. The highest concentration is closest to the biofilm in the epithelium where there is deregulation (inhibition) of the inflammatory and immune response which favours the invasion and persistence of *P.gingivalis*. In deeper tissues, their concentrations are reduced and the inflammatory response stimulated leading to the tissue destruction.

2.11. Animal models in periodontal disease

The use of animal models in medical and biotechnological research is controversial and divisive.This has led to comprehensive legislation limiting the use of animals in research which in the UK is embodied in the Animals (Scientific Procedures) Act 1986 (https://www.gov.uk/research-and-testing-using-animals#animals-scientific-procedures-act-1986) and in European Law under the 2010 Directive on protection of animals used for scientific purposes (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF). These acts define 'protected animals' as any living vertebrae excluding man (with the addition of the invertebrate species *Octopus vulgaris*) and as such are subject to these legislation if subjected to any scientific procedure that causes pain, suffering, distress or lasting harm. In addition to strict regulation of all scientific procedures and controlled licensing of projects and personnel involved in animal research the underlying ethos of these laws is based on the principle of the 3 Rs:

Replace; use of non animal experiments wherever possible

Refine; modification of experimental techniques to minimize the suffering to animals

Reduce; minimize the numbers of animals involved in a scientific procedure

The major issue is that no single animal model exactly replicates the pathogenesis of any disease in humans. This is especially true for periodontal disease where differences in the oral structures or host inflammatory and immune responses can be marked.

However, if we are to satisfy Socransky's[139] modified Koch's postulates any potential putative pathogen must be able to show animal pathogenicity and demonstrate a plausible biological mechanism (section 2.6). It is in this latter demonstration that animal models are most useful.

Different animal models have different advantages and disadvantages so selection of the appropriate model is key to extrapolating any results into the pathogenesis of human periodontal disease. Very well-defined questions can be asked of certain models that are well chosen and well controlled[252]. For example, the role of virulence factors such as the proteases of *P. gingivalis* in the induction and expression of certain pro-inflammatory cytokines such as IL-1 and TNFα in the pathogenesis of periodontal disease. However, probably the best we can hope for is that animal models provide data on biologic trends before we proceed onto human applications[253].

2.11.1. Non-human primates

There are huge advantages to the use of phylogenetically similar animals to humans in the exploration of the pathogenesis of diseases. This is especially true in periodontal disease because non-human primates have very similar oral structures to humans. Their dental anatomy is similar in the primary and secondary dentition with histologically identical orientation of collagen in the teeth supporting structures. In addition, they are susceptible to plaque and calculus accumulation with resultant gingivitis and naturally occurring periodontal disease[254]. Accelerated periodontal disease can be induced by the use of ligatures around the teeth to encourage plaque accumulation[255].

Non-human primates have also been used to investigate the microbiology of human periodontal disease. *Porphyromonas gingivalis* is not a part of the commensal microbiota of Cynomolgus (*Macaca fasicularis*) monkeys but inoculation experiments have shown that after 5 months the micro-organism is detectable in the plaque biofilms and there is resultant periodontal bone loss[256]. In these experiments 8 female Cynomolgus monkeys were allowed to develop plaque and calculus accumulations with resultant gingivitis. Microbiological sampling was performed to confirm there was no *P. gingivalis* (at the time of the study *Bacteroides gingivalis*) present in these animals with gingivitis. These animals were then divided into two groups and either treated with the antibiotic Rifampin or a placebo. After 8 weeks ligatures were placed around 4

posterior teeth to induce periodontitis. Following 20 weeks of induced periodontitis Rifampin resistant *B. gingivalis* was implanted by injection into the subgingival pocket of 2 of the ligated sites in each animal on 6 occasions over 2 weeks. The results (Table 2.7) showed that periodontitis was induced in the majority of sites/animals treated with placebo (10 of 16 and 3 of 4 respectively). In animals treated with the active antibiotic the monkeys were more resistant to ligature induced periodontitis (4 sites in one animal with the other 3 animals remaining unchanged). Following implantation of *B. gingivalis* (now *Porphyromonas gingivalis*) the majority of these sites that were resistant to the ligature induced periodontitis underwent periodontal breakdown (5 of 8) with only a few (2 of 8) of the sites experiencing breakdown in the placebo groups. Interestingly the majority (6 of 8) of the control sites in the Rifampin treated animals (i.e. ligated but not implanted) did not experience any bone loss and in the remaining 2 sites there was no further bone loss (above that which was ligature induced) following *B. gingivalis* implantation. *B. gingivalis* was able to be re-isolated from all the implanted animals.

These authors concluded that direct implantation with *B. gingivalis* was directly connected to the clinical characteristics of periodontal disease. This was despite implantation into the complex ecological background of the oral commensal microbiota and was able to produce a 'burst' of bone loss; the implication being that this micro-organism might be important in the progression of periodontal disease as well as its initiation.

Treatment	Max % *B. gingivalis* after implantation		Bone loss (maximum change in CADIA) after implantation	
	Site 1	Site 2	Site 1	Site 2
Placebo	Not detected	Not detected	0.0	0.0
Rifampin	Not detected	Not detected	11.6	2.5
Placebo	Not detected	6%	0.0	0.0
Rifampin	3%	4%	0.0	0.0
Placebo	Not detected	10%	0.0	15.4
Placebo	7%	13%	9.3	5.8
Rifampin	14%	18%	7.0	8.4
Rifampin	18%	27%	8.9	11.3

Table 2.7 Percentage of *B. gingivalis* (as proportion of overall cultivable bacteria) and bone loss (as maximum change in computer-assisted densiometric analysis from radiographs) detected in 2 sites with ligature induced periodontitis in cynomolgus monkeys treated with Rifampin antibiotic or placebo following *B. gingivalis* implantation into these sites by injection. Adapted from Holt et al[256].

Studies in non-human primates have also investigated the interaction between *P. gingivalis* and host inflammatory and immune response. Immunisation of Squirrel monkeys (*Saimiri sciureus*) with *P. gingivalis* has been shown to lead to elevated levels of anti-*P. gingivalis* IgG in the serum of infected monkeys[257] demonstrating an interaction of the bacterium with the adaptive immune system of the challenged animals. Antibody response to infection with *P. gingivalis* or its component antigenic structures has also been shown in Macques (*M fasicularis*). These studies immunised animals with either whole bacterial cells of *P. gingivalis*, *P. intermedia* or *B. fragilis*[258] or cell envelope or cell wall antigens[259] from Macque-derived *P. gingivalis*, *P. intermedia*, *F. nucleatum* and *C. rectus*. Antibody determinations were made using ELISA and inoculation with whole cells and antigenic components led to raised levels of IgG and IgA.

The non-human primate model has also been used to investigate the potential links between periodontal disease and systemic disease[260]. Specifically the translocation of oral bacteria into the blood resulted in alterations in serum lipids/lipoproteins. There were increased levels of endotoxin (LPS), acute phase proteins (e.g. CRP) and inflammatory cytokines (e.g. IL-8) in animals with experimentally induced periodontal disease. This has been followed up in later studies with reference to the acute phase proteins[261]. In these investigations non-human primate models were used in parallel with clinical data from patient oriented investigations. The main objective was to determine the effect of periodontal disease on serum acute phase proteins (APP) and to this end in the clinical arm the serum levels of APP were determined by ELISA in individuals with localised chronic periodontitis (LCP), generalised aggressive periodontitis (GAP) and Sjögren's syndrome (SS). In the animal part of this study cynomolgus monkeys had serum APP evaluated under normal conditions, after mechanical debridement, in gingivitis and in ligature induced periodontitis. In both LCP (once a threshold was reached) and GAP individuals there were increased serum APP, specifically C-reactice protein (CRP) and haptoglobin (for LCP individuals). The results from the animals showed decreased levels of serum APP following mechanical

debridement and elevated CRP and fibrinogen in ligature induced periodontitis. The conclusions were that periodontal inflammation can initiate or contribute to a systemic inflammatory response. This systemic inflammation offers a potential mechanism for the development of systemic diseases such as atheroma.

More recently non-human primates have been used to investigate the impact of nutrition on periodontal disease[262] where it was found that calorie restriction dampened the inflammatory response and led to reduced periodontal inflammation. In addition, this model has been used to investigate clinical applications such as the use of recombinant human derived growth and differentiation factor (rhGDF) in regeneration of periodontal defects[263].

The major disadvantage of the animal model is the difficulty and expense of husbandry often requiring specialist facilities; monkeys can be temperamental and vicious in nature. They are also susceptible to infectious diseases such as tuberculosis limiting their use[254]. Probably the main disadvantage is the ethical issues surrounding the use of this animal model system.

2.11.2. Miniature pigs

The Minnesota pig (minipig) was developed for biomedical research in the 1950s[264]. For oral and dental research they have similar oral structures to humans and develop oral diseases in a similar manner. Accumulation of plaque biofilms leads to gingival inflammation from 6 months of age which leads to periodontal inflammation with increasing age (16 months). The inflammatory infiltrate is histologically similar to humans[265]. Periodontal disease can be accelerated by the use of ligatures or with inoculations of periodontal pathogens such as *P. gingivalis*. Early interest in this model focussed on evaluation of soft tissue pedicle grafts[266], which are a means of covering exposed tooth root surfaces due to gingival recession (mucogingival defects), and the effects of inflammation on these mucogingival defects[267].

This model has been used extensively to investigate the use of regenerative procedures especially the use of autologously generated stem cells from the gingival tissues to repair surgically created periodontal defects[268]. Gingival margin derived stem/progenitor cells showed greater reduction in clinical periodontal indices when used to repair the defects in conjunction with various scaffold materials than the scaffold materials alone. Another group looked at similar periodontal ligament derived stem cells (PDLSCs) in the repair of surgically created and ligature induced periodontal defects[269, 270] and concluded that this cell lineage has potential for regenerative therapies.

The minipig has also been used to look at the treatment of peri-implant disease. Changes in the microbiology of ligature induced peri-implantitis have been investigated[271] where it was shown that there is a shift from Gram-positive facultative micro-organims to a microbiota consisting of Gram-negative obligate anaerobes including black-pigmenting *Bacteroides sp*. Surgical repair of peri-implant defects has also been examined in this model where 3 treatment modalities were investigated[272]; debridement of the implant surface, surgical coverage and surgical coverage with expanded polytetrafluoroethylene membrane (ePTFE). This latter group demonstrated the greatest degree of resolution of the lesion with some gain in bone height with some evidence of re-osseointegration. More recently the uses of composite graft materials have been investigated to augment the placement of implants into extraction sockets[273]. In this study polymethacrylate, polyhydroxymethacrylate, calcium hydroxide plus polyanhydride (PPCH-PA) was shown to result in greater stability of the implant in the extraction socket site along with well organised implant-bone interface allowing immediate loading of the implant.

The major disadvantages again relate to the expensive and space consuming husbandry needs of the minipig.

2.11.3. Dogs

The oral and dental anatomy of the canine is somewhat different from humans but they do suffer from naturally occurring gingivitis and periodontal disease. Dogs that are maintained under plaque free conditions by scaling do not develop gingivitis[274, 275]; it is only in the presence of a dental plaque biofilm that inflammation develops[276]. With cessation of oral hygiene measures, plaque develops in a similar succession pattern to humans with shifts in the composition from Gram-negative rods and cocci to the presence of more spirochetes in the later stages of inflammation[158, 277]. Evidence of periodontal bone loss is seen radiographically across a number of breeds of dogs[278]. There is however, huge variation between breeds and within the same breed as to susceptibility to periodontal inflammation which may be a function of the specifics of the infection or genetics[279].

The microbiology of the subgingival plaque in periodontal disease in the canine model is largely Gram-negative cocci and rods with *P. gingivalis* and *F. nucleatum* present in similar forms to the human strains[280]. There may however, be differences between the cultivable subgingival microbiota between humans and dogs. It has been reported that there is a higher proportion of Gram-negative bacteria in health in the dog when compared to humans. In addition, in the diseased state in the dog, there is an increase in *Prevotella melaninogenica* which is not mirrored in humans[281].

In addition, dogs have much faster bone turnover and different bone thickness and architecture limiting their use in regenerative studies[282]. There are also differences in the histological features of the inflammatory lesions between humans and dogs with the complete thickness of the marginal gingiva being affected in dogs rather than only that adjacent to the gingival pocket wall[283].

This model was used in the pioneering days of dental implants by Brannemark to show osseointegration of their implant system[284]. Dogs have also been used to investigate the effects of peri-implant disease and its treatment. Experimental peri-implantitis can be induced in a dog by ligature placement[285] and following removal of the ligature there is continued spontaneous progression of the lesion. The degree of re-osseointegration has been examined in this model with the degree of re-integration determined by the method of cleaning of the 'infected' implant surface[286] and the nature of the implant surface[287, 288].

Husbandry challenges include the need for space and exercise for the dogs as well as companionship, limiting their use in the study of periodontal disease.

2.11.4. Other animals

A number of other animals might be suitable for models of periodontal disease. Horses do show naturally occurring gingival recession and periodontal pockets which seems to increase with age[289]. However, husbandry is difficult due to their size and the space needed to house them making their use to date unpractical.

Domestic sheep suffer from a naturally occurring form of periodontitis termed 'broken mouth' which results in premature and spontaneous exfoliation of teeth[290, 291]. This has been proposed as a model for periodontal disease especially in rapidly progressing forms of disease where inflammatory and immune biomarkers (such as IgG to infecting micro-organisms) have been elucidated[292]. In addition, this 'broken mouth' model has been used to investigate the microbial aetiology of periodontal disease[293]. These workers compared the subgingival microbiota and sera of 8 healthy sheep and 8 sheep with 'broken mouth' periodontal disease. In most animals, healthy or diseased, pathogenic micro-organisms such as *P. gingivalis*, *B forsythus* and *P. intermedia* were detected but those with 'broken mouth' had elevated titres of IgG to these bacteria and their components such as fimbriae.

Again husbandry issues and expense may preclude their use. In addition with any higher vertebrate there are increased ethical issues.

Rabbits have been suggested as a useful model for periodontal disease especially as the microbiota associated with disease closely mirrors that in humans. Micro-organisms such a *Fusobacterium nucleatum*, *Prevotella heparinolytica*, *Peptostreptococcus micros,* Streptococcus milleri group and *Actinomyces israelii* were found by culture means in the rabbit: micro-organisms that are consistent with those found in the subgingival microbiota of humans with periodontal disease. This model has also been used to investigate the effects of *Porphyromonas gingivalis* induced periodontal disease[294]. One of the main areas of investigation using this model system has been the effect resolvins on the resolution of periodontal inflammation. These are bioactive products of omega-3 fatty acid products brought about by the action of aspirin (eicosapentanoic acids) through the cyclo-oxygenase 2 pathway (COX-2). In these studies, New Zealand white rabbits had ligature-induced periodontitis treated with topical application of eicosapentanoic acid-derived Resolvin E1 (EPA RvE1) and the results showed that as a therapy this offered protection against inflammation and reduced alveolar bone loss[295]. The precise signalling pathway was later elucidated by the same group in human cell lines[296]. These lipid mediators are now thought to play an active role in resolution of tissue inflammation[297] and have been proposed as a valid target for periodontal therapy based on the data derived from studies in the rabbit[298].

Ferrets naturally develop calculus and periodontal disease in a similar manner to humans[299]. Natural periodontal disease develops with the accumulation of plaque and calculus and they are also susceptible to ligature-induced periodontal lesions[300] which show similar histological features to human disease.

2.11.5. Rodent models

The use of rodent models to investigate periodontal disease stems from observations made in the 1940s. Keyes et al[301] noted plaque like accumulations around the gingival margins of the molar teeth of hamsters and that led to periodontal bone loss. Moreover, it was noted that this 'infection' was transmissible between hamsters resulting in bone loss and that certain strains of hamster (albino) were resistant to this process.

Rodents have only one continuously erupting incisor and 3 molar teeth per quadrant. The molar anatomy has a number of similarities to humans that make it appropriate as a disease model in that they are multi-rooted teeth with furcations. A number of different model systems within these animals have been used to explore various aspects of the pathogenesis of periodontal disease. In addition the development of germ free and genetically modified animals at relatively low cost means they are a burgeoning area for modelling the pathogenesis of periodontal disease.

2.11.6. Rat

Rice rats (*Orzyzomys palustris*) are swamp dwelling rats that are endemic to the South of the United States of America. Early observations showed these are very susceptible to periodontal disease [302, 303] and that it appeared to be dependent on nutrition[304]. The transmissibility of periodontal disease in rats is explained by coprophagia; the consumption of faeces[305]. Although very different microbiological niches, the oral and gut microbiota in rats have a number of common component micro-organisms including micro-organisms such as *Acinetobacter sp* and *Lactobacillus sp*[306]. Consequently the oral micro-organisms find their way into the gut microbiota and ultimately are found in the faeces. As a result of coprophagia there exists a circular route for re-infection of the oral cavity with these oral (and gut) micro-organisms. The histopathological features of periodontal disease in the rat have been described and show some similarities with humans[307]. Culture of *Streptococcus sanguinis*, *Actimomyces* sp and *Lactobacillus* sp have been found associated with the oral

microbiota at 5-9 weeks old, an age when these animals start to develop periodontal disease[308].

The development of gnotobiotic (animals in which only known micro-organisms are present) rats pushed the frontiers of medical and periodontal research further. Mono-infection of these rats with filamentous (*Actimomyces naeslundii*) micro-organisms derived from human isolates were able to cause periodontal destruction[309]. However, infection with Gram-negative rods, derived from human individuals with localised juvenile periodontitis (now localised aggressive perodontitis) such as *Actinobacillus* (now *Aggregatibacter*) *actinomycetemcomitans,* did not lead to formation of a plaque biofilm[310]. More recent studies have shown that the human periodontal pathogens *P. gingivalis* and *T. forsythia* can induce an immune response and periodontal breakdown and that there may be increased bone loss in rats infected with mixed consortia of the 2 micro-organisms[311].

Concurrent with this was the development of germ free rats. This model has been used to investigate the effects of infection with *P. gingivalis* on periodontal bone loss[312] along with structural components of this micro-organism such as inactivated fimbriae[313]. Germ free rats infected with a mutant strain of *P. gingivalis*, without the *fim*A gene that encodes the major fimbriae, were less virulent in terms of periodontal bone loss suggesting a pivotal role for the fimbriae in mediating bacterial attachment and periodontal pathogenesis.

It has been argued that the rat is a limited model for periodontal research as periodontal disease is different from that in humans, occurring with very rapid periodontal breakdown following disease initiation[253]. In addition, periodontal breakdown can occur without apical migration of the junctional epithelium[314]. Probably the most useful application for this model is in investigating alterations in the oral microbiota.

The rat model has also been extensively used in studies looking at periodontal regeneration. In one such study, the use of recombinant human bone morphogenetic protein (rh BMP-2) was used in the repair of surgically created defects in Wistar rats[315]. These authors found that when compared to sham treated controls the rats treated with BMP-2 developed increased cementum formation but levels of collagen regeneration were similar and overall there were no differences in longer term healing. These results have been confirmed by other authors using similar rat models[316]. Using the same surgical defect rat model, other groups have looked at the potential benefits of collagen membranes[317] and polytetrafluoroethylene (PTFE) membranes[318, 319] in the repair of these defects and showed enhanced bone formation in the early phase of healing. The use of enamel matrix proteins has also been evaluated in this model with regard to wound healing[320, 321] where they have been shown to enhance periodontal healing and reduce post-operative recession. Other more novel regenerative techniques have been investigated in this rat model, such as the use of fibronectin matrix based multi layered sheets of fibroblasts, which showed enhanced alveolar bone regeneration in the surgical defect[322]. This use of periodontal cell lines as regenerative therapy has been investigated more recently with the use of periodontal ligament cells and gingival fibroblasts. PDL cells showed increased and more functional regeneration of bone and ligament fibres[323].

2.11.7. Mouse

Mice have been used in periodontal research for more than 50 years largely because of their ease of handling, minimal husbandry needs, prolific breeding, suitability for genetic modification and availability of germ free strains.

Many strains of mice have been used in periodontal research so it is important to know how these perform as a model of periodontitis and more specifically how one of the main signs, alveolar bone loss, is manifest[324]. In this study, the authors investigated the genetic susceptibility of different mouse strains to *P. gingivalis* induced alveolar bone loss. The results showed that among the most susceptible mice were Balb/c mice whilst the more resistant strains included C57BL mice (Figure 2.12).

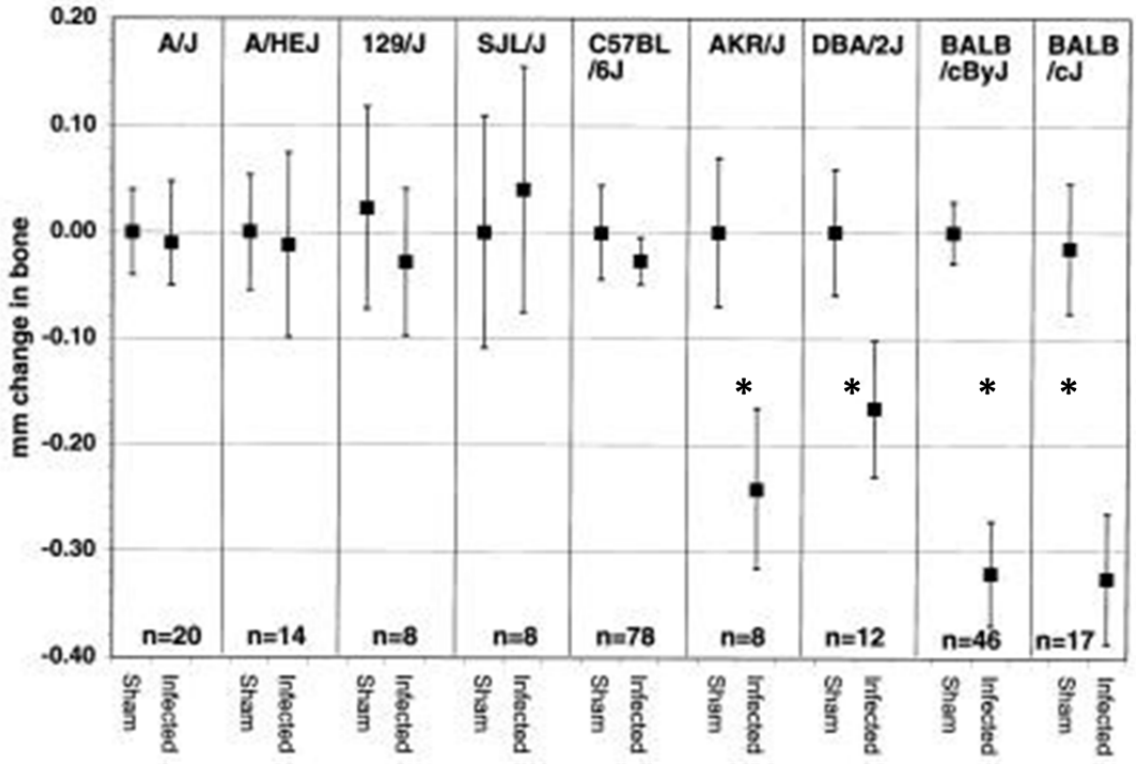


Figure 2.12 The effect of *P. gingivalis* oral gavage on alveolar bone loss in different strains of Specific Pathogen Free (SPF)mouse. Bone loss is represented as a negative value +/- standard deviation relative to a baseline value which represents the alveolar bone level of a control (sham infected) A/J mouse. * represents statistically significant differences between controls and inoculated mice at a value of $p<0.05$. From Baker et al 2000[324].

2.11.7.1. The specific pathogen free (SPF) mouse

The original concept of specific pathogen free rodents was developed in the 1960s by Russell Schaedler to address the huge variability in the commensal microbiota of experimental mice. He colonised germ free mice with a mixture of known micro-organisms derived from normal mice[325]. This mixture was then supplied commercially to other investigators to colonise their germ free mice colonies with a view to providing a standard mouse model[326]. This mixture was later modified by the National Cancer Institute and termed the 'altered Schaedler flora'[327]. Later molecular categorisation of the 8 bacterial strains in this altered flora has given a higher level of detail of the infecting micro-organisms (Table 2.8)[327]. In addition this has been shown to be stable for specific suppliers of these mice[328].

Species	ASF Strain	Original Schaedler Flora	Altered Schaedler Flora
Escherichia coli	-	+	-
N Streptococcus group	-	+	-
Enterococcus faecalis	-	+	-
Clostridium sp	356	+	+
Lactobacillus sp	360	+	+
Lactobacillus murinus	361	+	+
Flexistripes group	457	-	+
Eubacterium plexicaudatum	492	+	+
Low G-C content Gram + group	500	-	+
Clostridium sp	502	-	+
Bacteroides sp	519	+	+

Table 2.8 List of the bacterial strains contained in the original Schaedler flora (OSF) and those in the altered Schaedler flora (ASF) along with the current strain identification. Adapted from Stehr et al [328].

The composition of the microbiota of SPF mice has changed over the years. Specific pathogen free (SPF) mice are now considered mice that are demonstrated to be free from a specific list of pathogens as determined by routine testing. The list includes disease causing pathogens as well as opportunistic and commensal micro-organisms that do not cause disease. In general, these micro-organisms can vary between different mice dependent on the barrier level of the room in which they are housed. It can also vary between suppliers e.g. The Jackson Laboratory have a list of agents it monitors (http://jaxmice.jax.org/health/agents_list.html) which is different to those monitored by Charles River International (http://www.criver.com/SiteCollectionDocuments/hmsummary.pdf). Typically, however, there is a degree of consensus about which micro-organisms are excluded for an animal to be termed specific pathogen free (Table 2.9).

Virus	Bacteria	Parasites
MHV (mouse hepatitis virus)	*Mycoplasma pulmonis*	Endo parasites
MVM (minute virus of mouse)	CAR bacillus (cilia associated respiratory bacillus)	Pinworms (*Syphacis sp, Aspiculuris tetraptera*)
GDVII (mouse encephalomyelitis virus)	*Citrobacter rodentium*	Tapeworms (*Hymenolepis sp*)
EDIM (enteric disease of infant mice)	*Salmonella spp*	Protazoa (*Giardia muris, Encephalitozoon cuniculi*)
PVM (pneumonia virus of mice)	*Clostridium piliforme*	Ectoparasites
LCMV (lymphocytic choriomeningitis virus)		Mites (*Myobia musculi, Myocoptes musculinus, Radfordia affinis, Psoregates simplex*)
MCMV (mouse cytomegalovirus)		
Hantaan virus		
Sendai virus		
MPV (mouse parovirus)		
Reovirus-3		
K virus		
Ectromelia virus		
Polyoma virus		
Mouse adenovirus		
MTV (mouse thymic virus)		

Table 2.9 List of micro-organisms in specific pathogen free mice that are routinely tested for and excluded. List courtesy of University of Chicago, Illinois, USA (http://oicf.bsd.uchicago.edu/docs/MouseSpecificPathogenFree_Exclusion_List-1.pdf)

2.11.7.2. The aging mouse

A number of mouse models of periodontal disease have been used. Mice naturally develop periodontal bone loss at around 9 months of age and this then increases as the mouse ages[329]. Early studies of aging and periodontal bone loss suggested that the observed phenomena of increasing alveolar bone loss with increasing age might be a manifestation of physiological and functional changes brought about by tooth movement[330]. This was further looked at in a senescence accelerated mouse (SAM-P/2/Iw) and a senescence accelerated resistant mouse (SAM-R/1/Iw)[331]. Neither strain exhibited chronic periodontitis and no naturally occurring plaque or calculus was found. However, in both strains ~15% alveolar bone loss was found at 13 months of age around the third molar and this continued with increasing age. The bone levels of the first molar were lower (increased bone loss) in the age accelerated mutant from 1 month and this was maintained as significantly increased for the lifespan of the mouse. These results show that alveolar bone loss is a natural physiological process of aging in the mouse.

To further test the observation that there is increased alveolar bone loss with increasing age, a model was proposed that used a surgical procedure to induce the bone loss in animals of differing ages[332]. Muco-periosteal flap surgery (MFS) was perfomed on the buccal aspects of the mandible in 72 CF1 mice aged 3, 6, 9 and 12 months. The mice were also divided into 3 groups; male, breeding (non-virgin) females and non breeding (virgin) females to elucidate the role any hormonal differences may play. The contralateral mandible acted as controls. 21 days following surgery alveolar bone loss was assessed and it was found that the surgical sites had increased bone loss in all groups compared to control side. Differences in bone loss were significantly larger in 3 and 12 month animals compared to 6 and 9 month. From these data it was proposed that the 3 month old CF1 mice be a useful model for 'provoked' alveolar bone loss assessment.

In a more recent study of the aging mouse differences in alveolar bone levels of Balb/c mice at a range of ages from 8-10 week (young) to ≥ 18 month (old) were assessed[333]. In addition, the expression of pro-inflammatory cytokines was examined. Alveolar bone loss occurs gradually but slowly until around 9 months of age when there is a dramatic increase in further bone loss up to the age of 18 months. Using a qPCR technique the differences between pro-inflammatory cytokines was examined between young and old mice. There were significant elevations in IL1β, TNFα and IL17A in the older animals as well as expression of inflammation inducing or amplifying receptors TLR2, CD14, CD11b, CD18, C5a and TREM1. The conclusion was that mice develop periodontal bone loss as a function of aging.

The role of the macrophage in aging has been looked at in the mouse model[334]. In this study macrophages were harvested from young (8-10week) and old (≥18 month) Balb/c mice. These macrophages were then activated with *P. gingivalis in vitro* and age related alterations assessed. In the aged macrophages certain receptors that amplify inflammation were elevated, namely C5a anaphylotoxin receptor and triggering receptor expressed on myeloid cells (TREM-1). There was no observed decrease in the older macrophages in terms of phagocytosis and intracellular killing of *P. gingivalis* consistent with no changes in expression of phagocytic receptors. The conclusion was that the macrophage function does not decline with age.

Recent interest has centred on developmental endothelial locus 1 (Del1) which is a naturally occurring inhibitor of neutrophil extravasation[335]. For a coordinated and effective inflammatory and immune response neutrophils must be able to cross from the lumen of a blood vessel, extravasate into the tissue and migrate to the site of infection. This extravasation requires a coordinated cascade that involves interaction with integrins such as LFA1. Del 1 inhibits this process by interfering with LFA1. Del 1 is reciprocally cross regulated with the pro-inflammatory cytokine IL17. In aging mice the levels of Del1 were decreased, with reciprocal increased expression of IL17, and this was associated with increased periodontitis with increasing age. Del 1 deficient mice,

missing the inhibitor of neutrophil extravasation, develop spontaneous periodontitis at young ages with the features of excessive neutrophil infiltration (a function of the loss of Del1) and increased expression of IL17. When given as a local agent Del1 inhibited this IL17 increase, the neutrophil accumulation and ultimately led to decreased alveolar bone loss.

2.11.7.3. The oral gavage model of experimental periodontitis

This model was developed in the 1990s and is sometimes named after its developer; the Baker Model[336]. In this model mice are initially given antibiotics to suppress the commensal oral microbiota and thereby ostensibly allow the test infecting agent a foothold in the oral cavity. This improves the reliability and reproducibility of the resultant periodontal bone loss[337]. The mice are then infected via the oral cavity with a known periodontal pathogen or antigen. Inoculation takes place on 3 separate occasions 48 hours apart and usually consists of a test micro-organism or component antigen suspended in a transport medium of 2% carboxymethyl cellulose (CMC). Mice are then sacrificed after 6-10 weeks and alveolar bone loss analysed by morphometric analysis or micro-computed tomography (micro-CT). The original methodology involved bone loss assessment of the maxillary molars as these were described as the first areas to show periodontal bone loss with bone loss reportedly developing slower in the mandibular molar regions. The continuous erupting incisors are not used for alveolar bone loss evaluation.

This model has been used to investigate the role of a number of suspected periodontal pathogens in the pathogenesis of the disease. Inoculation of C57 black mice with *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (*Aa*) was carried out according to this model and the effects on the expression of pro-inflammatory markers of disease was examined using real-time polymerase chain reaction (qPCR) and enzyme-linked immunoabsorbant assays (ELISA)[338]. The inoculation was accompanied by widespread migration of leucocytes into the gingival tissues and alveolar bone resorption. Two distinct patterns of expression of inflammatory cytokines were found.

Firstly, expression of specific matrix metallo-proteinases (MMPs 1, 2 & 9) and receptor activator of nuclear factor kappa-B ligand (RANKL) was correlated with expression of Interleukin 1 beta (IL1β), tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) and this was associated with an intense inflammatory reaction and alveolar bone loss. Secondly, expression of Interleukin 4 (IL4) and Interleukin 10 (IL10) were associated with higher expression of tissue inhibitors of metalloproteinases (TIMPs 1, 2 &3) and osteoprotegrin (OPG), lower expression of RANKL and MMPs with resultant lower cellular infiltration of the tissues with leucocytes and less bone loss.

Tanerella forsythia, and specifically its cell surface and secreted protein (BspA), have been investigated using this model[339]. Inoculation of Balb/c mice was with wild type *T. forsythia* or a genetic mutant defective in the expression of BspA protein. Following inoculations bacterial whole cell antibodies to the wild type and mutant micro-organisms were detected by ELISA and anti-BspA antibodies were only detected in animals inoculated with the wild type strain. Animals inoculated with the wild type showed increased periodontal bone loss when compared to sham inoculated control whereas those inoculated with the mutant strain had no significant bone loss compared to controls. The conclusion was that the protein BspA was a significant virulence factor for *Tannerella forsythia*.

The animal form of *Porphyromonas gingivalis* has been investigated in this mouse model system. *Porphyromonas gulae* is a Gram-negative, black pigmenting, obligate anaerobe, asaccharolytic, non motile rod that can be isolated from the gingival sulci of various animals[340](bear, cat, dog, wolf, coyote & monkey).This novel species was proposed following comparison of isolates from these various animals, which had been previously classified as *P. gingivalis*, with human strains of *P. gingivalis*. Following DNA-DNA hybridisation, 16S rRNA sequencing and biochemical assays it was proposed that there were enough differences in the animal derived strain from the human for a new species; *P. gulae*.

The aim of another study was to immunise the mice with inactivated cells of *P. gulae* and, following establishment of a robust antibody response, challenge the mice by oral gavage with live *P. gulae* and assess the degree of protection offered by the vaccine[341]. Mice immunised with *P. gulae* showed higher titres of anti-*P. gulae* antibodies and decreased alveolar bone loss.

The oral gavage experimental model of periodontitis has been used to investigate the feasibility of using components of pathogenic micro-organisms such as *P. gingivalis* as vaccines. One area receiving particular interest as a potential is the use of the gingipains RgpA and Kgp. These are extracellular and cell surface bound cysteine proteases that are important virulence determinants of the micro-organism. In one early study[342] immunisation of Balb/c mice was carried out using heat killed whole cells of *P. gingivalis* or with purified gingipains (RgpA & RgpB). Only mice immunised with whole cells or RgpA were protected from periodontal bone loss in the maxilla. All groups showed elevated levels of anti-*P. gingivalis* specific IgG. The potential of these virulence factors has been further elucidated in a later study[343]. Mice were immunised with either the RgpA-Kgp complex or purified adhesion binding motifs (ABMs) which had been developed synthetically and are areas of the RgpA-Kgp complex that are responsible for binding to host proteins. Both protected against periodontal bone loss when these animals were inoculated by oral gavage with *P. gingivalis*. The conclusion was that following immunisation with the RgpA-Kgp complex or a functional binding motif there was a Th2 immune response that was protective against periodontal bone loss by blocking this complex. In these studies there is no information on the degree of colonisation of the oral cavity of the mouse with *P. gingivalis* following inoculation.

The role of the fimbrial components of *P. gingivalis* in the pathogenesis of periodontal tissue have been investigated[344] where it was shown that strains of the micro-organism lacking in the components *fim*CDE failed to interact with CXCR4 receptors. There was resultant down-regulation of the inflammatory and immune response and loss of virulence of the micro-organism.

Using a similar oral gavage protocol mice were inoculated with *P. gingivalis* or *F. nucleatum* and bone loss assessed after 6 weeks. Lower levels of alveolar bone were recorded in those mice infected with these micro-organisms compared to controls[345]. There were also increased levels of pro-inflammatory cytokines such as Interleukin 1 (IL1) and Tumour Necrosis factor alpha (TNFα) in infected mice.

Other peridonto-pathogenic micro-organisms such as *A. actinomycetemcomitans* have been examined in this model. In one study[346] gene regulation of an auto-inducer protein was investigated. One method of inter-bacterial communication within a biofilm is quorum sensing (see section 2.8.4.2.) which is a cell-density dependent response mediated by small signalling molecules called autoinducers. These authors found that de-activation of the autoinducer gene (AI-2) led to decreased virulence.

Aspects of the host immune signalling have also been tested in this model, specifically the role of Toll-like Receptor 2 and 4 (TLRs2&4). Mutant C57 mice were bred with defects in the gene that codes for TLR2 and TLR4, the mutant mice being completely deficient in these receptors[347]. These mice were immunised in a subcutaneous chamber (see section 2.11.7.6) with *P. gingivalis* and following examination of the expression of inflammatory cytokines they were repeatedly challenged by oral gavage with live *P. gingivalis*.TLR4 deficient mice and wild type mice showed elevation in expression of pro-inflammatory cytokines following immunisation, namely IFNγ, IL1β, IL10 following immunisation. TLR2 deficient mice did not express increased levels of IFNγ and IL10. In terms of periodontal bone loss following repeated oral gavage the wild type and TLR4 deficient mice showed significantly increased levels of bone loss when compared to controls. In contrast, there was no difference between TLR2 deficient mice and controls. The fact that TLR2 deficient mice were more resistant to periodontal bone loss following oral challenge with *P. gingivalis* highlights a potential role for this receptor system in the mechanism of periodontal bone loss.

The effectiveness of this model has been compared to ligature induced periodontitis in which a silk ligature is tied around the molar teeth to encourage plaque biofilm formation and induction of inflammation. In this study periodontitis was induced either by oral gavage or ligature soaked in *P. gingivalis* ATCC33277[348]. It was found that ligature soaked in *P. gingivalis* induced greater bone loss and increased osteoclast numbers than oral gavage or ligature induced periodontitis alone over the 67 day experimental period. There were also elevated levels of pro-inflammatory cytokines such as Interleukin 1 beta (IL1β) and Interleukin 6 (IL6) in the group with *P. gingivalis* soaked ligature induced periodontitis.

Much interest has been stimulated in recent years about potential associations between periodontitis and systemic disease. This model has offered the opportunity to explore these links mechanistically. In one study[349] C57 black mice, with a mutation in apolipoprotein E (*ApoE*), were repeatedly (15 times) inoculated with *P. gingivalis* 381 or sham inoculated over a 3 week period. The mutant mice spontaneously develop hypercholesterolaemia which leads to atherosclerotic lesions. During the experiment, serum was taken for analysis of lipoprotein and upon sacrifice the aorta were dissected out and examined for atherosclerotic lesions. The mice challenged with *P. gingivalis* developed larger atherosclerotic lesions with detection of the micro-organism within the lesion in 2 of the 9 animals tested. The concurrent presence of periodontal disease was confirmed by assessment of alveolar bone levels at sacrifice and elevated expression of pro-inflammatory cytokines. In a further study similar ApoE null mice were inoculated with wild type *P. gingivalis* and a fimbriae deficient mutant of *P. gingivalis* (*fimA*). In this case both wild type and mutant *P. gingivalis* was detected in the atherosclerotic lesion of the aorta by PCR, however, the mice challenged with the mutant strain did not develop concurrent periodontal disease. The atherosclerotic lesions in the mice challenged with wild type *P. gingivalis* were more extensive and there was upregulation of TLR2 and TLR4 in this tissue The conclusion was that *P. gingivalis* accelerates the development of atherosclerotic lesions in a highly susceptible (*ApoE* deficient) mouse.

2.11.7.4. Severe Combined Immunodeficient (SCID) and Non-Obese Diabetic (NOD) mice

The original oral gavage studies involved severe combined immunodeficient (SCID) mice[336]. These mice have a mutation in the gene *scid* which affects lymphocyte development: the hypothesis being that if T and/or B cells were implicated in periodontal breakdown animals with the *scid* mutation should be protected (as the T and/or B cells are defective). It was found that significant periodontal bone loss did still occur in the mutant mice, lacking B and T lymphocytes, when compared to sham infected immunocompetent SCID mouse controls or uninfected immuno-competent mice. Hence they concluded that neither T nor B cells are absolutely essential for periodontal bone loss induced by oral gavage with *P. gingivalis*.

Non-obese diabetic (NOD) mice are initially healthy mice that are rendered diabetic with the drug streptozotocin which is toxic to pancreatic β cells. In the pioneering study using this model[350] these mice were then inoculated with *P. gingivalis*. Bone loss assessed at 2 and 3 months after inoculation was increased in the diabetic animals along with increase Advanced Glycation Endproducts (AGE) and expression of its receptor (RAGE) in the gingival tissues. Other groups have used this NOD model to unravel aspects of the inflammatory and immune response in periodontal disease including the transplantation of human peripheral blood lymphocytes (HuPBLs) from human individuals with periodontal disease into NOD mice[351]. Infection of these transfused mice with *A. actinomycetemcomitans* led to activation of CD4+ cells and alveolar bone loss. Similarly bone marrow derived macrophages taken from NOD mice, when stimulated with the *P. gingivalis* antigen; lipopolysaccharide (LPS), under hyperglycaemic conditions, exhibited reduced production of TNFα and expression of Toll-like Receptor 2 (TLR2) with resultant reduced phagocytic function. They concluded that in the hyperglycaemic state, individuals may be producing macrophages that are functionally compromised contributing to the increase tissue breakdown seen.

2.11.7.5. Murine abscess model

In this model a subcutaneous injection is made, usually to the dorsal region of the mouse with a periodontal pathogen or its antigens. The mice then develop a lesion at the site of the injection as well as secondary lesions at various distal sites[252]. The main use for this model is to investigate the immunologic potential of whole cells or bacterial components and it has also been used to extensively assess the virulence potential of different bacterial strains or combinations of these potentially virulent micro-organisms.

Immunisation with *P. gingivalis* derived polysaccharide offers a degree of protection to mice that are subsequently infected with *P. gingivalis*[352]. This immunisation with polysaccharide partially reduces the formation of primary and secondary abscesses but does not offer complete protection. The potential synergistc effects of polymicrobial infections have also been examined in this model[353]. Mono infection with *P. gingivalis* and *T. denticola* produced a level of infection which was lower than when they were combined for injection; the microbial complexes exhibiting enhanced virulence.

The arginine specific gingipains of *P. gingivalis*, specific cysteine proteases that are suspected virulence factors, have also been investigated using this model. Double mutants defective in the two gingipains have shown decreased abscess formation and virulence potential[354]. In addition, this model has been used to develop a potential vaccine to *P. gingivalis*. Injection of mice with Rgp (gingipain)-derived from recombinant DNA offered protection from infection with *P. gingivalis*[355].

Further investigations of the gingipains of *P. gingivalis* have used this murine virulence model. In one such study the effectiveness of a protease inhibitor of Kgp was examined[356] and its efficacy as a protease-inhibitor based therapy evaluated. This inhibitor was found to limit the growth of wild type *P. gingivalis* W50 (forming a phenotype similar to Kgp mutant strains) and these bacterial cells were significantly less virulent in the mouse abscess model.

The conclusion was that Kgp was confirmed as an important factor in terms of both nutrition and virulence of *P. gingivalis* and that protease-inhibition of Kgp may offer a novel targeted therapy.

This model has also been used to elucidate the immune cellular response to infection with periopathogenic micro-organisms. Immunisation with *P. gingivalis* led to a predomination of acid phosphatase-positive and phagocytic macrophages which the author suggested is consistent with a strong T cell mediated immune response[357]. In a later study in mice with lesions induced by the injection of *T. forsythia,* the immune response was stronger than for injection of non-viable micro-organisms. There was evidence of an adaptive T cell response by day 7[358].

2.11.7.6. Subcutaneous chamber model

This model utilises the surgical placement of a small, usually titanium, chamber in the subcutaneous tissues in the dorso-lumbar region of the back of the mouse. This offers a contained environment into which micro-organisms and their antigens can be injected to test the host inflammatory and immune response. This system has the advantage of allowing repeated sampling of the ongoing host response from within the chamber at sequential time points[359].

This model has been used to look at the role stress plays in the aetiology of periodontal disease[360]. Mice were immunised with *P. gingivalis* and also received local challenge within the chamber with *P. gingivalis*. The mice were subjected to stressful conditions (isolation and restraint); one group during the immunisation and the other during the local challenge. Chamber exudates and serum were analysed at a number of time points to assess levels of antibodies (IgG, IgG1 and IgG2a) against *P. gingivalis*. In the stressed groups there was a marked increase in the levels of antibodies in the chamber at day 7 suggesting stress played an important role in modulation of the immune response at a local level.

Investigations of the links between periodontitis of the mother and pregnancy outcomes, specifically pre-term low birth weight, have been tested using this model[361]. In this study Balb/c mice were inoculated with heat killed *P. gingivalis* into the subcutaneous chamber and mated 2 weeks later.The rationale behind pre-treating the mice with heat killed *P. gingivalis* is that it offers protection from host clearance of subsequent inoculation into the chamber with live *P. gingivalis*. After 7.5 days gestation the pregnant mice were challenged with a live *P. gingivalis* broth (or sham control) and sacrificed at day 16.5 gestation. The foetuses were then assessed for foetal growth restriction (FGR) defined as a weight that was 2 standard deviations smaller than controls. Of the 20 challenged mice, 8 had FGR foetuses in their litters and in the dams of these mice there were PCR signals for *P. gingivalis* within the liver and uterus in 3 cases. In addition, there were elevated levels of serum antibodies to *P. gingivalis*, TNFα and IL-10. The conclusion being that FGR is associated with dissemination of the micro-organism throughout the dam and modulation of the host inflammatory and immune response.

Specific components of the host inflammatory and immune response have also been looked at in this model in combination with SCID mice[362]. In this study the chamber model was used to test the hypothesis that lymphocytic cells regulate the monocytic hyperinflammatory trait seen in individuals with periodontitis. The mice were reconstituted with peripheral blood mononuclear cells from human donors with periodontal disease. They were then challenged in the chamber with *P. gingivalis* and samples from the chamber taken after 7 days. The levels of Prostaglandin E2 (PGE_2) were examined by enzyme-linked immunoabsorbant assay (ELISA). The results showed that significantly elevated levels of PGE_2were found in patients with moderate/severe periodontitis compared to mild periodontitis/gingivitis and this resulted in lymphocytic sensitisation. The conclusion was that the hyperinflammatory response in moderate/severe periodontitis is in part due to lymphocytic modulation.

This model has also been used to examine the virulence of specific periopathogenic micro-organisms. Following oral inoculation with *P. gingivalis* and/or *F. nucleatum*, sampling of a subcutaneous chamber was performed to assess the inflammatory and immune response; specifically the levels of TNFα and IL1β[345]. This study found that mixed infection produced greater levels of bone loss and greater elevations in both pro-inflammatory cytokines than mono-infection with either species. In a later study using a similar model these findings were further tested by inhibiting the co-aggregation of these 2 micro-organisms with lactose[363]. The hypothesis being that co-aggregation of the bacteria is required for the increased virulence. This was somewhat challenged by the results which showed that inhibition of co-aggregation actually led to increased periodontal bone loss when compared to un-aggregated mixed infection. The co-aggregated infection also slightly attenuated the inflammatory response through examination of the levels of TNFα and Il1β. The conclusion was that a mixed infection might be more representative than a co-aggregated infection and resultant periodontal bone loss may be more due to the substantivity of *F. nucleatum*.

The same investigators have also used this model to examine the virulence of different strains of the same micro-organism[364]. Mice were orally infected with *P. gingivalis* ATCC33277, 53977 and 381 and the subcutaneous chamber used to monitor the inflammatory and immune response through levels of anti-*P. gingivalis* IgG1 and IgG2a and inflammatory cytokines. The greatest alveolar bone loss was in the group infected with *P. gingivalis* 53977 and this correlated with elevations in the pro-inflammatory cytokines but lower in the humoral response.

2.11.7.7. Chemically induced mouse model

A recent development in the murine model involves inducing inflammation in the gut with trinitrobenzene sulphonic acid (TBNS) or dextran sulphate sodium (DSS)[365, 366]. This methodology has been used for some time in research into inflammatory bowel disease (IBD) where these chemicals are used to induce acute and chronic inflammation[367, 368]. There is evidence that the induced inflammation is a function of the commensal microbiota in the gut[369]. DSS is given orally and acts by undermining the epithelial barrier and is an immune cell activator leading to innate immune tissue breakdown. TNBS was given rectally and appears to function as a hapten which modifes autologous proteins and induces a T cell (auto-immune like) response[370].

The induced colitis in this model has been associated with general bone loss[371]. DSS treated Balb/c mice exhibited significantly lower femoral bone mass and micro-architecture as determined my micro-computerised tomography (Micro-CT). There was an osteopenia associated with increased numbers of osteoclasts and reduced bone formation that correlated with the degree of gut mucosal inflammation.

As a model for periodontal disease the use of these chemicals has been investigated[365, 366]. Treatment of Balb/c mice with oral TNBS twice a week or DSS given in their feed for 18 weeks produced increased periodontal bone loss in both groups. The oral treatment with DSS led to systemic complications such as colitis and hepatic changes whereas oral treatment with TNBS produced no systemic effects. Both compounds had local effects on the oral tissue. The conclusion was that oral administration of these chemicals produces alveolar bone loss in conjunction with the commensal oral microbiota.

2.11.8. Knockout mice

Knockout mice, mice with specific genes 'turned off', were pioneered in the late 1980s by Capecchi, Evans and Smithies for which they shared the Nobel Prize for Physiology in 2007. In short, they developed a method of homologous recombination of selected DNA sequences into the mouse chromosomal DNA (gene targeting) in pluripotent mouse embryonic stem cells[372]. These stem cells could then be used to generate mice of any desired phenotype by injection of the mutant embryonic stem cells into mouse blastocysts and then interbreeding of heterozygous siblings to produce the desired phenotype. Searching the suppliers database shows just how many genetic variations are now commercially available (Jackson Laboratory, USA, http://jaxmice.jax.org/query/f?p=205:1:2057614857768198:::::, and Charles River International, http://www.criver.com/en-US/ProdServ/ByType/ResModOver/ResMod/Pages/ResModels.aspx).

For the purposes of this thesis this review of knockout mice will be limited to mice that have given insight into the recognition molecules for alveolar bone loss (TLR-2, TLR-4 and Nod-1) and the specific knockout mice used in the experiments reported on in this thesis (CXCR2 knockout).

2.11.8.2. TLR knockout mice

Toll-like receptors (TLRs) are transmembranereceptors expressed on host cells (gingival epithelial cells, fibroblasts, monocytes/macrophages and neutrophils)[373] and are highly evolutionarily conserved . These form part of the innate response to bacterial challange and they recognise distinct pathogen-associated molecular patterns (PAMPs) such as LPS, lipoproteins and fimbriae from bacterial cells. Activation leads to recruitment of a cytoplasmic adaptor molecule, myeloid differentiation primary response 88 (MyD88), and finally activation of nuclear factor kappa B (NF-κB) which is a protein that controls DNA transcription and mitogen-activated protein kinases (MAPK) which regulate cell differentiation and mitosis[374]. Activation leads to up-regulation of the genes associated with inflammation and this is a driving factor in periodontal disease[347] .

Intracellular recognition of bacteria seems to involve a TLR-independent system in which the nucleotide oligomerization domain (Nod) family of proteins play an important role[375]. Nod 1 and 2 recognise motifs found in the layer of peptidoglycan (γ-D-glutamyl-mesodiaminopimelic acid; iE-DAP) and the muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP) respectively. MDP is found in most bacteria whereas iE-DAP is limited to Gram-negative bacteria hence it is through these that Nod 1 and 2 play a role in recognition of bacterial infection inside the host cell (Figure 2.13).

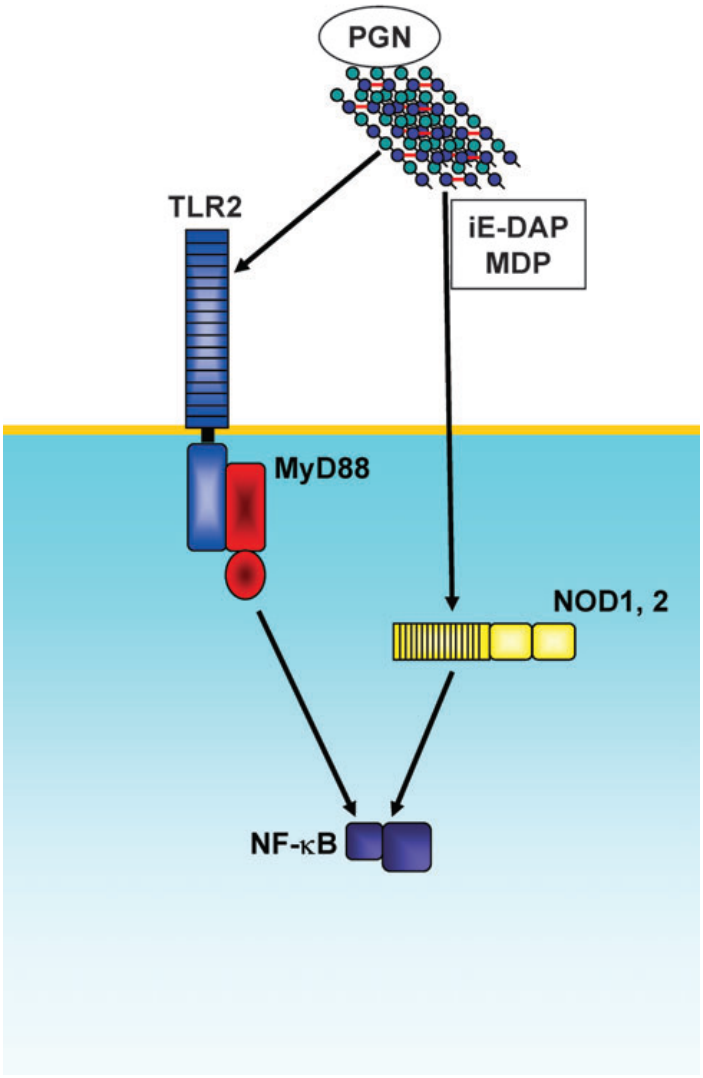


Figure 2.13 Activation of a transmembrane TLR (in this case TLR-2) on the cell surface of a host cell leads to induction of MyD88 and NF- κB with upregulation of pro-inflammatory cytokines. There is also a pathway in which intracellular activation of the Nod 1 and 2 by bacterial peptidoglycan (PGN) leads to up regulation of pro-inflammatory cytokines. Image from Takeda et al 2005[375].

The evidence for the activation of the toll-like receptors by PAMPs as a driving factor in periodontal inflammation was discovered and elucidated using knockout mice. Some early studies focussed on TLR 4 and looked at a mixed anaerobic infection with *Prevotella intermedia*, *Fusobacterium nucleatum*, *Streptococcus intermedius* and *Peptostreptococcus micros* into the exposed pulp chamber of a molar tooth in a genetic mutant of a C3H mouse that was TLR-4 deficient[376]. After 21 days the mice were sacrificed and periapical bone levels assessed. It was found that the mutant mice, deficient in TLR-4, had less periapical bone loss than the wild type. This correlated with lower levels of bone resorptive cytokines such as IL-1α, IL-1β and pro-inflammatory cytokines such as IL-12. The conclusion was that the increased pro-inflammatory response and bone destruction in wild type mice in response to mixed anaerobic infections was mediated through TLRs.

Other workers have investigated the response through the TLRs to antigenic components of known periodontal pathogens in a knockout mouse model with defective TLRs. In one such study the activation of murine macrophages was investigated by *Treponema denticola* and its major outer sheath protein (MSP)[377] or lipo-oligosaccharide (LOS). These studies used C57 black mice that were wild type, TLR-4 deficient or TLR-2 deficient. It was found that *T. denticola* and its MSP were able to stimulate innate immune responses through TLR-4/MyD88 whereas LOS acted through TLR-2/MyD88. These results suggested there was flexibility in pattern recognition of this micro-organism but that MyD88 was a requirement downstream of whichever TLR was activated.

This signalling pathway has been further investigated using knockout mice, deficient in MyD88 and TLR2/TLR4. A soluble heat shock protein 60 (HSP60) had been previously shown to activate innate inflammatory responses through TLR2. This TLR2 activation of human T cells by HSP60 led to increased adhesion to fibronectin with decreased chemotaxis, down-regulation of pro-inflammatory IFN-γ and TNF-α and enhanced IL-10 secretion from activated T cells. This protein was used to treat cell lines from the knockout mice (B and T cell deficient) and it was found that the effect of this protein was largely through TLR-4 and MyD88 signalling, cells from the deficient mice showing decreased response to HSP60[378]. This was followed up by the same group using knockout mice for TLR-2, MyD88 and TRIF (an additional signalling protein). In these mice the subcutaneous chamber methodology was used to assess the responses of each type of mouse to challenge with *P. gingivalis* 381. The results suggested that pro-inflammatory cytokine production was dependent on TLR-2 activation but did not require MyD88. In contrast, the generation of an intracellular oxidative burst and clearance of *P. gingivalis* required MyD88.

Other authors have confirmed the importance of TLR-2 for an innate response to micro-organisms such as *P. gingivalis* using knockout mice. In one study TLR-2 and TLR-4 knockout mice were challenged with live *P. gingivalis* 381 via a subcutaneous chamber and innate immune response and bacterial survival investigated. It was found that wild type and TLR-4 deficient mice produced high levels of inflammatory cytokines in response to the challenge with *P. gingivalis* but in TLR-2 deficient mice the cytokine levels were much lower. Surprisingly, *P.gingivalis* was cleared much more easily in these TLR-2 deficient mice and in addition they were much more resistant to alveolar bone loss following subsequent oral challenge with *P. gingivalis*.

2.11.8.3. Nod knockout mice

Intracellular recognition of bacteria is a function of a TLR independent system that involves a family of proteins termed nucleotide binding oligomerisation domain (Nod) 1 and 2. Initial interest centered upon the ability of these proteins to detect unique muropeptides from Gram negative bacteria[379]. Development of Nod deficient mice led to the availability of cell lines from these mice that could be used to further elucidate the role of these proteins. In one such study[380] the macrophages from Nod1 deficient mice were found to be unable to secrete inflammatory cytokines in response to stimulation with synthetic bacterial-like peptidoglycan (iE-DAP). Other authors have used a Nod2 deficient mouse to show that in the gut the protective mechanisms that result from the recognition of bacterial muramyl peptidoglycan by Nod2 is abolished in the knockout mice and that these animals are susceptible to bacterial infection by this route[381].

Recent studies have investigated the effect of mutations in the Nod1 gene and alveolar bone loss in the mouse model[382]. Using mice deficient in the Nod1 these investigators induced periodontal inflammation by means of a ligature tied around the molar teeth. The findings showed that mice deficient in Nod1 had reduced bone loss and impaired neutrophil recruitment and osteoclasts into the gingival tissues. Interestingly these authors also found that a specific murine oral commensal bacteria (NI1060 and sharing around 60% sequence homology with *A. actinomycetemcomitans*) accumulated at the site of ligature placement and that this, when examined *in vitro*, was a powerful activator of Nod1 and brought about neutrophil recruitment and bone resorption.

It can be seen from the evidence presented in the previous 2 sections that TLR2 and Nod1 deficient mice are resistant to *P. gingivalis* induced alveolar bone loss whereas TLR4 deficient mice do not appear to show this resistance.

2.11.8.4. CXCR2 knockout mice

These mice were developed by identification of the part of the murine genome that had high homology with the human genes for the receptors for IL-8. Deletion of these mouse genes and recombination in embryonic stem cells allowed subsequent interbreeding to produce mice deficient in the receptors (CXCR2) for the mouse homologues of IL-8; CXCL1 and CXCL2[383]. These mice are outwardly healthy but suffer from lymphadenopathy due to accumulations of B cells, splenomegaly due to accumulations of neutrophils and metamyelocytes and they also experience spontaneous periodontal bone loss.

The use of these knockout mice in periodontal disease research has been limited to date. One group looked at mice that were deficient in the receptor for the pro-inflammatory cytokine TNF-α[384]. These mice (in C57 black background) were challenged by oral gavage with *A. actinomycetemcomitans* JP2 clone (a strain that has been strongly associated with aggressive periodontal disease in a cohort of the population of West African origin). Deficient mice developed less severe alveolar bone loss and reduced inflammatory response following challenge with *A. actinomycetemcomitans* than wild type but this impaired protective immunity resulted in increased bacterial load. Interestingly they also exhibited lower levels of the murine chemokine CXCL1 and lower expression of its receptor CXCR2. It was concluded that this impaired protective response resulted in reduced chemotaxis of neutrophils, lymphocytes and macrophages into the gingival tissues.

In another study primarily concerned with elucidating the role of IL-17 in the recruitment of neutrophils into inflamed periodontal tissue the CXCR2 knockout mouse was also used[385]. The line of enquiry involved oral challenge of IL-17 deficient mice with *P. gingivalis* A7A1-28 and examination of the migration of neutrophils into the gingival tissues but CXCR2 knockout mice were also challenged in a similar manner. These authors concluded that, because the CXCR2 knockout mice exhibited severe alveolar bone loss without *P. gingivalis* challenge, that CXCR2 may play an important role in bone homeostasis. The neutrophils in these mice exhibit normal killing activity but have impaired transmigration into the site of inflammation. Ultimately, these results suggested that a CXC chemokine deficiency was associated with a phenotype that exhibited more severe bone loss than the wild type or IL-17 knockout mouse.

2.11.9. Hamsters

Periodontal disease in hamsters was first described in the 1950s[386] where the lesions were characterised in Syrian hamsters (*Mesocricetus auratus*) and later the transmissibility of the disease between animals was investigated[387] and the nature of the disease process[388]. Similarly periodontal bone loss has been described and quantified in these hamsters[389].

This model has been used to investigate the effects of pathogenic bacteria on the pregnancy outcome in a chamber model similar to that of mice. Live or heat-killed *P. gingivalis* was injected into these animals[390] on the 8th day of gestation and foetal weight and viability assessed. Those hamsters challenged with live *P. gingivalis* (+/- heat killed bacterial cells) had elevated levels of TNFα and PGE_2 at day 5 and these elevations were associated with foetal growth retardation and embryo-lethality; an adverse pregnancy outcome. Further experiments tested a similar set of parameters following inoculation with *E. coli* and *P. gingivalis* LPS[391]. Administration of both these antigens was significantly associated with adverse pregnancy outcomes.

2.11.10. Conclusions

From this review of the use of animal models in periodontal research it can be seen that no single model is ideal, each having its own advantages and disadvantages. The main reasons for choosing the oral gavage mouse model were as follows.

Firstly, it is an ideal model for studying the effects of *P. gingivalis* as this micro-organism is not a part of the murine commensal microbiota. The pathogenicity of *P. gingivalis* in this model has been well established. In addition, we were able to select a desired commensal microbiota and maintain control of this throughout the various experiments.

Secondly, control of mice with respect to age and genetic background is relatively simple and achievable within an acceptable time frame due to rapid breeding. The availability of mice with specific genes 'knocked out' is an additional benefit as well as the relative simplicity of developing a germ free colony.

Finally, one of the biggest advantages is the ease of handling and low cost of these animals. Husbandry is straight forward with simple nutritional needs and day/night cycles.

There are, however, some limitations which must be considered in interpreting any results derived from this model. The oral cavity is small making sampling and inoculation challenging. In addition, the dental anatomy is slightly different from humans (constantly erupting incisors and different crown and root morphology for molar teeth). It is also worth noting that there are differences in the immune response to challenge with *P. gingivalis* with the predominant cellular response being reported as lymphocytic compared to a neutrophil response in humans.

All things considered the oral gavage model of experimental periodontitis in the mouse was considered an appropriate system to investigate the influence of the commensal oral microbiome in periodontal disease.

2.12. Experimental determination of periodontal disease

We have selected to use the oral gavage model of experimental mouse periodontitis which has been shown to produce measurable bone loss[336] and has a high degree of reliability[93 324 350]. In addition, this model produces measurable bone loss when challenged with a variety of human periodontal pathogens[338 339] as well as animal periodontal pathogens[341] in a reasonable time frame and at acceptable cost (see section 2.11 for more detailed discussion). The next issue to address is as to the most appropriate methodology for measuring this experimental periodontal disease.

There have been a number of methodologies proposed for measuring periodontal disease in experimental animal models each with their own advantages and disadvantages. Early investigators used a combination of techniques to examine the periodontal tissues. For example one group of authors looked at periodontal disease in the rice rat[303]. These authors investigated three techniques used on the heads fixed in alcohol and examined under 30x magnification but looking at more macroscopic features. Firstly, they examined the extent of the soft tissue involvement by rating the colour, contour and consistency of the gingival tissues. These were assigned an arbitrary value from 0 to 4; a 0 score indicating no detectable abnormalities through to a score of 4 indicating severe detachment of gingival tissues and recession frequently with food impaction between the teeth and mobile teeth. Secondly, evaluation of bone loss using a method described by another group[386]. In short, this involved the measurement of the distance between the alveolar bone crest (ABC) and the cement-enamel junction (CEJ) in the areas adjacent to the cusps of the molar teeth using a graduated blade (0.2mm intervals) and expressing the distance as a function of these (0.2mm) units; units of bone resorption. Thirdly, they assigned arbitrary units of bone loss from 0 to 4 where a score of 0 represented no detectable bone resorption and a score of 4 represented severe resorption with no remaining bone supporting the teeth. They concluded that the second method of bone loss was most accurate and that the evaluation of the soft tissues was the poorest experimental measureof periodontitis.

2.12.1. Histological measurement of periodontal disease

The use of histological assessment of the periodontal tissues has been advocated as a means of measuring periodontal disease in animal models. Early studies[392] focussed on infection of gnotobiotic rats with specific micro-organisms and determination of periodontal disease by measurement of the plaque, the apical migration of the epithelial attachment and bone loss as determined by demonstrating the presence of osteoclasts on the alveolar bone surface by means of acid phosphatase staining. The results from this study showed that the gnotobiotic rats developed periodontal disease in response to the 3 infecting micro-organisms (*A. naeslundii*, *A. viscosus* and *S. mutans*) when measured in this way.

Age-related histological changes in the tissues of the periodontium have been investigated in germ free rats[393]. These authors examined the periodontal tissues at 10 day intervals for germ free Sprague-Dawley rats aged between 30 and 80 days. They found that there was increased distance between the ABC and the CEJ at certain sites (lingual side lower 1st molar and lingual/palatal side of upper and lower second molar), there was sporadic migration of the junctional epithelium and that there were variations in the density of gingival fibroblasts but the overall concentration of collagen remained constant.

Detailed description of the histological changes that occur following infection with *A. naeslundii* especially in terms of osteoclasts have been published[394]. Five week old germ free rats were sacrificed at sequential time points following infection with *A. naeslundii* and the tissues fixed with glutaraldehyde and decalcified with EDTA. There were differences noted between the rats maintained in germ free conditions and those infected with *A. naeslundii*, notably in the degree of inflammatory infiltrate in the lamina propria and overlying epithelium with mainly neutrophils. There were also changes in the epithelium with apical migration of the junctional epithelium, development of more prominent rete ridges and evidence of ulceration. In addition, there was evidence of bone resorption in the infected rats with surface scalloping of

the alveolar bone surface evident and observation of osteoclast cells. With increasing age these features were more exaggerated.

These studies give detailed descriptions of the histological changes that occur with the initiation and progression of periodontal inflammation but as a measure of experimental periodontal disease they have drawbacks. The major issues relate to lack of quantification which is an inherent part of a measure of any disease. There is a degree of subjectivity in relation to the amount of inflammatory changes that are seen and consequently the severity of the disease. In addition, this methodology requires tissue sections (typically 1µm thick) which only offer a snapshot of the condition of the tissue as a whole.

2.12.2. Radiographic measurement of alveolar bone loss

Histological studies showed that there was frequently alveolar bone loss, especially in the interproximal areas, and that these areas were often obscured by the bulk of the facial or oral alveolar bone present; these infrabony defects often missed by direct observation. One method of overcoming this would be by the use of radiographic techniques.

In an early study germ free rats were either infected with *A. viscosus* or maintained in germ free conditions and rats from each group were examined at 4 different time points following infection (days 26, 46, 60 and 88)[395]. The defleshed jaws were radiographed using a standardised technique and the anatomical landmarks of the alveolar bone crest and cement-enamel junction marked. Bone loss was assessed by 3 techniques: the area between these landmarks for individual molar teeth, mean vertical distance between these landmarks and the deepest distances at predetermined sites that included the interproximal areas. The results supported the use of radiographic techniques to assess alveolar bone levels, especially interproximally, as accurate, sensitive and reproducible.

Radiographic assessment of alveolar bone loss has also been used in studies to assess the effects of specific (*Serratia marcescens*) endotoxin and ligature-induced periodontal inflammation in Sprague-Dawley rats[396]. 2 groups of rats were used; one group had a non-specific immune response induced by the endotoxin (endotoxin tolerant) the second group had not been challenged with the endotoxin. It was found that the endotoxin-tolerant rats exhibited significantly lower levels of alveolar bone loss. This bone loss was assessed by plain radiographs and was measured between the alveolar bone crest (ABC) and cement-enamel junction (CEJ) using a magnifying glass with built in calibrated scale measuring at 0.02mm. Alternatively, the radiograph can be projected and measurements made at a more macroscopic scale and converted to the representative distance as long as the scale factor of the magnification is known or a magnified scale is used (Figure 2.14) and this has been used in a study of the effects of chlorhexidine mouthwash in controlling gingivitis and periodontitis in dogs[397].

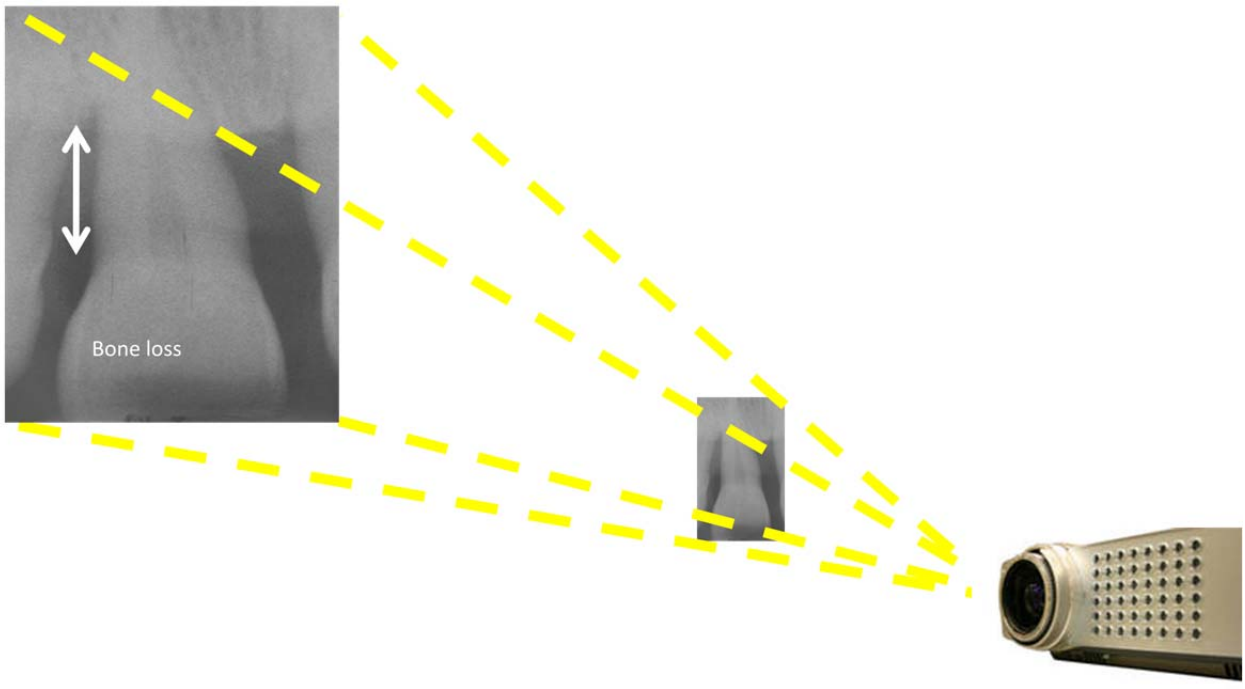


Figure 2.14 Radioraphic determination of alveolar bone levels using a projection technique.

2.12.3. Impression technique for measuring alveolar bone loss

Some workers have advocated the use of dental silicone impression materials to fill the periodontal defects which can then be accurately trimmed to the exact dimensions of the defect and volume measurements performed by water displacement to quantify the alveolar bone loss[398]. However, this technique has not been widely used.

2.12.4. Micro-computed tomography to measure alveolar bone loss

With recent advances in imaging technology the use of micro-computed tomography (Micro-CT) has become increasing used in the analysis of periodontal bone loss. This involves the taking of regular and serial images of a rotating sample with a fixed x-ray source (Figure 2.15). The images (slices) are then reconstructed using computer software to render a 3-dimensional image of the sample.

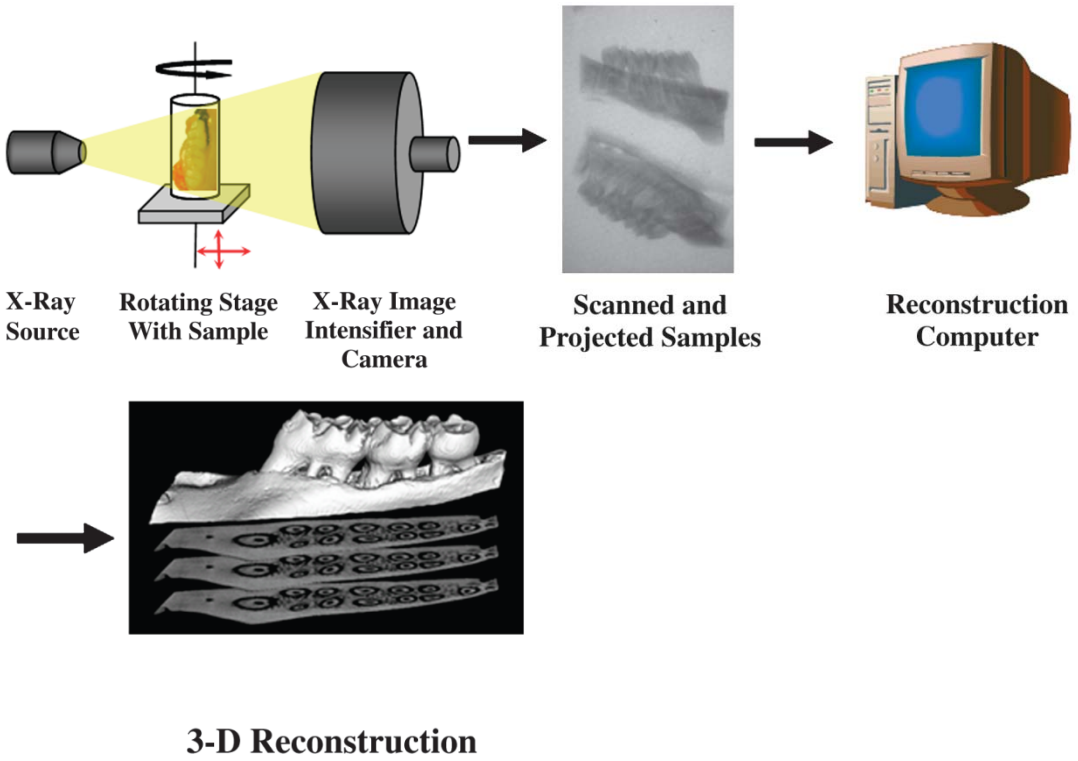


Figure 2.15 The stages in cone beam Micro-CT assessment of alveolar bone loss. The x-rays pass through a rotating sample and are captured by a camera producing a series of 2-dimensional slices that are reconstructed into a 3-dimensional image by computer software[399].

The major advantage of this methodology is that it can be used to visualize and measure infrabony defects that may be missed when plain radiographs are used (Figure 2.16). However, scans are expensive and time consuming meaning the use for numerous samples can become prohibitive.

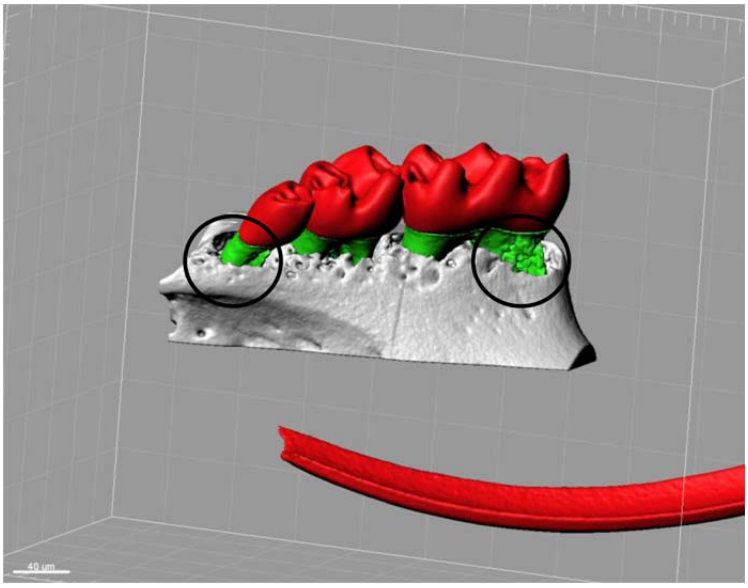


Figure 2.16 3-D reconstruction of mouse mandible using Micro-CT with different tissues rendered in different colours based on their relative radio-opacities (enamel is red, dentine is green and bone is grey). By rotating the image, defects that are contained within the bone (infrabony defects) and would be maksed by plain radiography can be visualised and measured (circled area). Scans performed by Graham Davis, Queen Mary University of London using the MuCAT scanner (http://www.dentistry.qmul.ac.uk/staff-list/50-readers/155-davis-graham) and image analysis using IMARIS software (http://www.bitplane.com/go/products/imaris).

It is not surprising that Micro CT has become the new gold standard for assessing periodontal bone loss in experimental animals. It has been shown to be highly reliable and reproducible[399]; these workers compared micro-CT with other more conventional methods of determining alveolar bone loss in rats following induction of inflammation with *P. gingivalis* LPS and subsequent regenerative therapy. Linear and volumetric parameters were compared and intra-operator and inter-operator reliability was highly reproducible and reliable.

Micro-CTs have also been performed on live specimens[400] which allow sequential scans to be performed offering valuable data at different time points with the potential to reduce the number of animals required (other techniques require sacrifice of the animals at sequential time points to assess bone levels). These workers investigated the effect of a novel phospholipase A_2 inhibitor on alveolar bone loss and found that as a therapeutic agent this inhibitor had no significant effect on periodontal bone loss. However, as a means of assessing bone levels, live micro-CT was found to correspond well to area measurements under stereo-microscopy (morphometric technique).

2.12.5. Morphometric analysis of alveolar bone loss

This technique was pioneered in the 1980s in Buffalo, New York[401] but was a development of much earlier techniques that used micrometres to measure the distance between the cement-enamel junction (CEJ) and alveolar bone crest (ABC) at points that corresponded to the cusps of the molar teeth[386]. It involves preparation of samples by removing the flesh from the jaws by boiling in water and physically macerating the tissues away from the underlying bone. The jaw bones are then positioned and carefully oriented under a microscope where direct measurements of bone loss can be made (Figure 2.17).

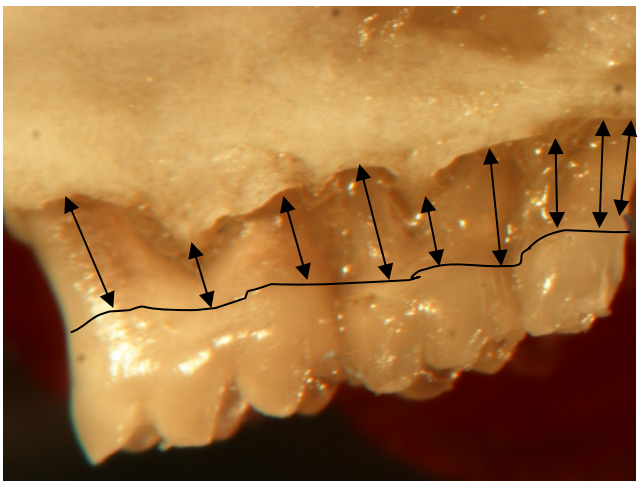


Figure 2.17 Morphometric analysis of alveolar bone levels using direct measurement of bone level as the distance from the Cemento-Enamel Junction (CEJ) to the Alveolar Bone Crest (ABC) at various points around the molar teeth of the mice.

Variations of this technique have been used. For example, in a study of transgenic rats with a defect in a component of the major histo-compatibility complex (HLA-B27) which led to impaired antigen presentation and consequently generalised severe inflammatory reactions[402]. These authors looked at the manifestations of this exaggerated inflammatory response by assessing periodontal bone loss. The bone loss was analysed by calculating the area of bone loss as defined by specific anatomical landmarks; mesially and distally by a line connecting the CEJ and ABC, coronally by 3 points on each molar tooth corresponding to the CEJ at mesial and distal most points and a further point at the apical most part of the CEJ, apically by the most apical position of the alveolar bone crest on each root surface (Figure 2.18). This has been used by other groups investigating aging and periodontal bone loss[332].

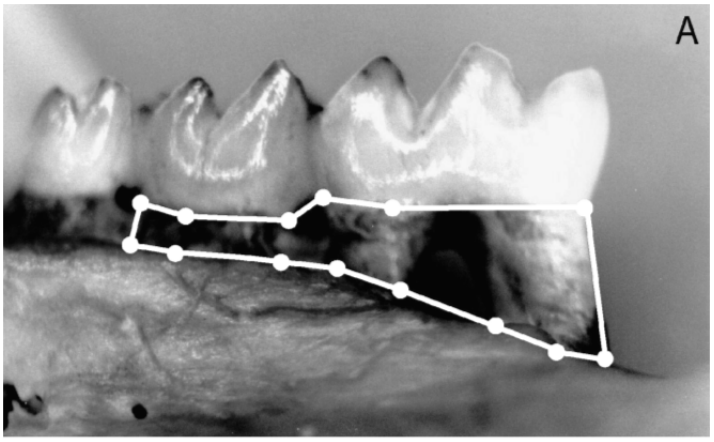


Figure 2.18 Morphometric assessment of alveolar bone loss based on area measurements using defined anatomical landmarks on the molar teeth[332].

Morphometric analysis is the most widely used and economical so was the technique that we used in the studies presented in this thesis. There are however, specific considerations that we will now discuss in more detail that must be considered with this technique.

2.12.5.1. Sample Preparation

The original protocol called for defleshing of the dissected jaw bones by boiling for 5 minutes then mechanically defleshing. They are then stored overnight in 1% Hydrogen Peroxide before staining for 1 minute with 1% Methylene Blue to demarcate the Cemento-enamel junction; an important landmark for measuring bone height. There have been variations on this technique such as boiling for 5 minutes at 15 p.s.i in a pressure cooker to improve the ease with which the soft tissues can be separated from the bone[403].

Using the original protocol of boiling the samples it quickly became apparent that the process was rather damaging to the fragile jaw bones. The result was that the jaw bones, especially the maxilla, would fracture and that could result in tooth loss. The fragments also became difficult to orient once the anatomical landmarks were lost. The result was that from the initial group around 20% of the data was lost. It may have been that with further development of knowledge in handling the tissues this might have been overcome but it was decided to investigate alternative techniques.

An alternative technique for macerating the samples was found. This involved the use of enzymatic digestion of the soft tissue using a proprietary detergent containing alkaline bacterial pronase (Terg-A-Zyme)[404]. The whole jaw (separated into whole maxilla and left and right mandibles) samples were incubated at 50^{o}C in a 4% solution of Terg-A-Zyme for 3 days. A degree of trial and error was needed with the initial samples as if they were immersed for too long in the enzyme solution (greater that 3 days) the teeth became loose and were ultimately shed. If the immersion time was too short (less than 2 days) the soft tissue had not softened enough to allow complete removal. The optimum time was determined as 3 days allowing enough softening of tissue to allow complete removal but with only minimal loosening of the teeth. There was still enough loosening of the teeth to allow displacement within the tooth socket which had a knock on effect when performing the morphometric analysis; the tooth

had to be fully seated in its socket to ensure the position of the CEJ relative to the ABC was maintained.

2.12.5.2. Microscope

Materials and equipment varied between the studies using this technique but the optimal magnification is of the order of 20-40x. In some studies the bone level is measured directly through a scale in the eyepiece[401] , in others an image is captured and analysed on a computer screen using image analysis software. This had the advantage of saving the image for future reference and use as well as allowing measurement to the limit of 1 pixel of resolution.

In this study we used 25x magnification on a Stemi SV11 dissecting microscope (Zeiss, Germany) and image capture facility (Nikon). The images were analysed using Image J software (National Institute of Health, USA, http://rsb.info.nih.gov/ij).

2.12.5.3. Orientation of sample

The positioning of the sample on the table of the microscope was vital to ensure the accuracy of the measurements. The original protocol[401] called for the sample to be oriented such that the buccal and lingual cusps are superimposed i.e. the sample is perpendicular to the line of sight. If the sample was tilted the measurements will be increased or decreased depending on direction of tilting (Figure 2.19).

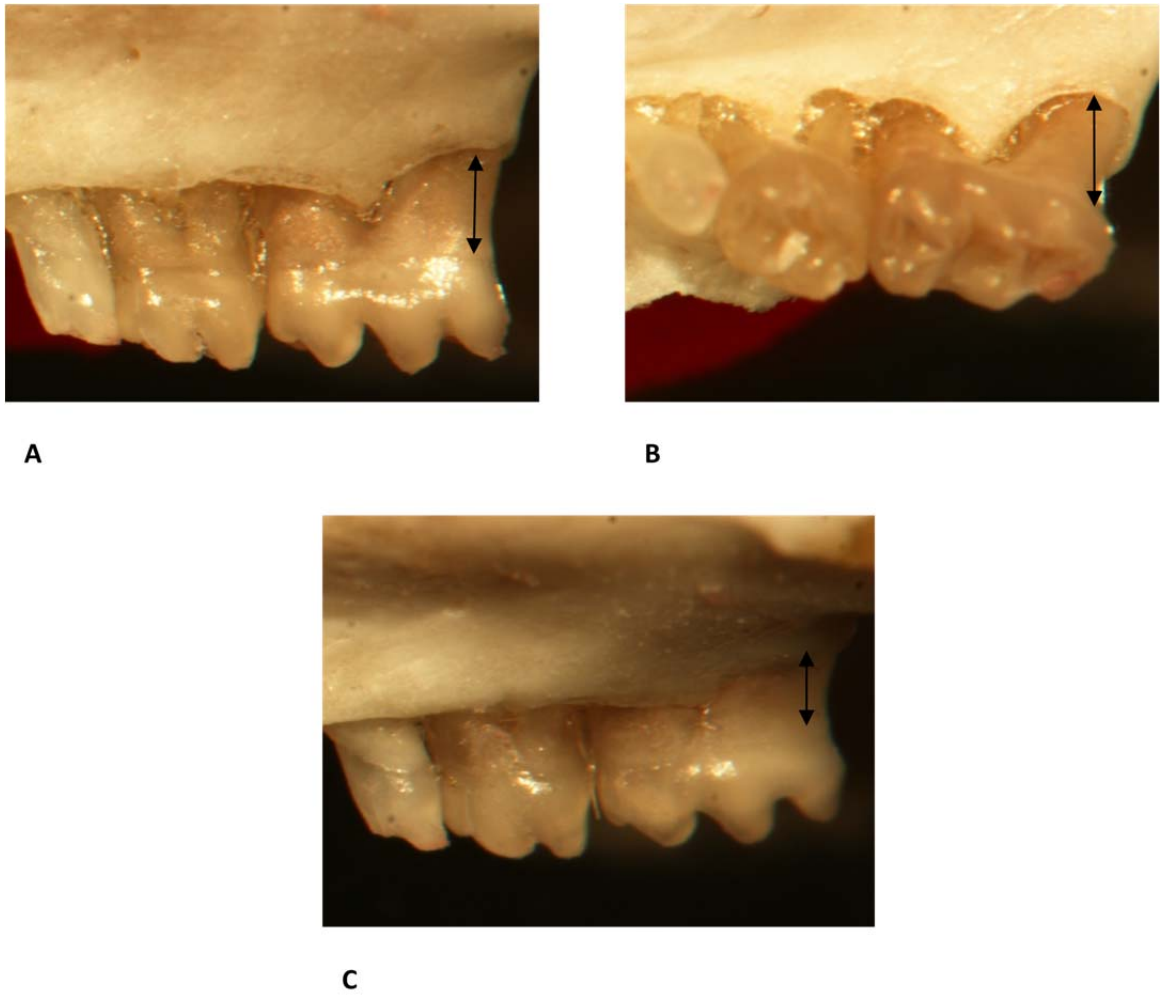


Figure 2.19 Correct orientation of the sample perpendicular to the line of sight (A) is vital to ensure that there was accurate measurement of the distance from the CEJ to the ABC. Tilting of the sample can significantly affect the measurements; tilting toward the view piece can elongate measurements (B) whereas tilting away can shorten measurements (C).

Studies have used custom made holders[405] to orient samples for analysis of bone loss but in this study we chose to use dental wax as it allowed easy manipulation of the samples.This had the advantage of being stable enough to hold the sample in a fixed position but flexible enough to allow fine adjustment of angulation to achieve optimal orientation.

2.12.5.4. Points of measurement

There was wide variation in the literature on which point and even which jaw, the bone loss measurements are carried out. The original technique[401] measured 12 points on the buccal surfaces of all teeth in both jaws. Other workers[336] in this area measured 14 points on the buccal surfaces of maxillary molars only. This latter method has been the standard to date and used in the most recent studies in this area[385] (Figure 2.20).

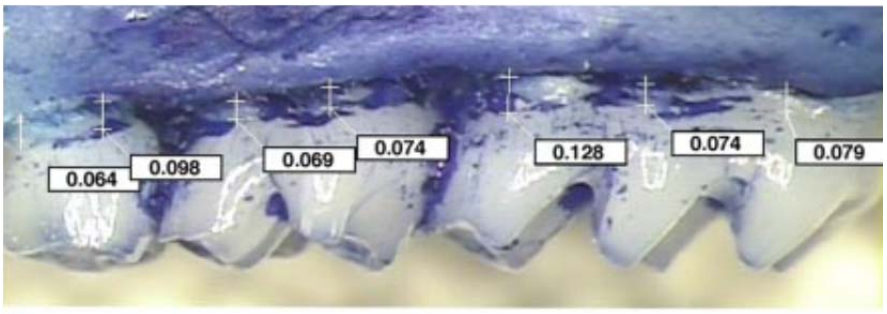


Figure 2.20 Standard sites for bone loss assessment corresponding to the cusp tips of the molar teeth and following defleshing and staining of the jaw with methylene blue[385].

In the studies presented in this thesis a combination of techniques has been used. The limitations to the accuracy of this technique using 14 (7 left side and 7 right side) points on the buccal side of the maxilla are discussed later. It was therefore decided to maximise the number of measurement taken to fully utilise the tissue available (maxillae and mandibles) and give a large pool of data that might minimise the inherent inaccuracies within this technique when the data was averaged out per group.

Clinical measurement of attachment loss in human subjects is based upon taking measurements of the probing depths of the periodontal pockets at 6 points around each tooth; Mesio-buccal (MB), buccal (B), disto-buccal (DB), disto-lingual (DL), lingual (L), mesio-lingual (ML) (Figure 2.21).

Following examination of the images captured in this study it was apparent that the majority of the bone loss occurred on the mesial root and especially in the mandible (Figure 2.22). This bone loss was likely to be missed using the original protocol (measuring the buccal maxilla only). It was therefore decided to use these clinical parameters to generate points to measure bone loss.

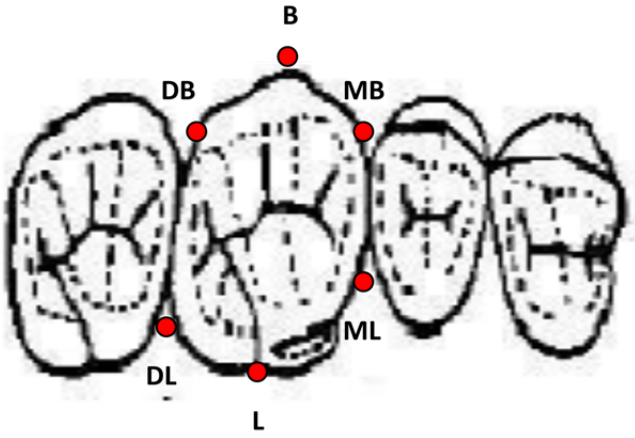


Figure 2.21 Position of the 6 points of measurement of probing depth around a tooth used to measure clinical indices in human subjects; mesio-buccal (MB), mid buccal (B), disto-buccal (DB), mesio-lingual (ML), mid lingual (L) and disto-lingual (DL).

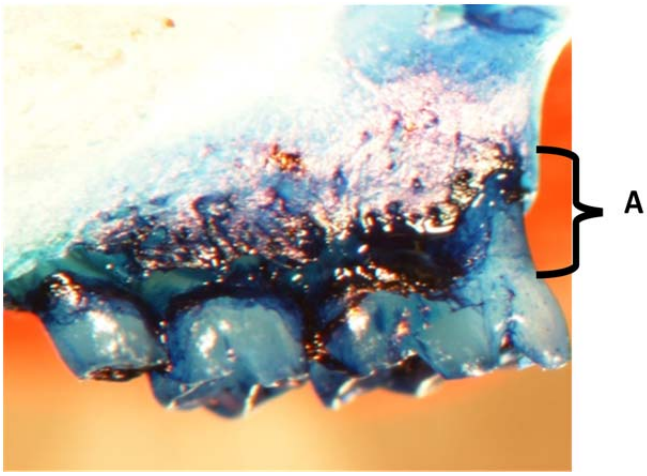


Figure 2.22 In the C3H and Balb/c mice used in these studies the most significant area of alveolar bone loss was found to be related to the mesial aspect of the first molar tooth (A).

Using this technique the number of individual measurements per mouse was increased producing a dataset that was on average 60% greater than using the standard protocol. These data were then pooled to produce a mean bone loss for each individual mouse.

2.12.5.5. Bone loss data

There is a distinct lack of conformity in the literature when it comes to expressing bone loss data. In the original study[401] bone loss was expressed as units of bone loss where 1 unit was equal to 0.37mm. This was a manifestation of the scale in the eyepiece and the smallest unit the authors were able to measure. Other authors have expressed bone loss as a percentage of relative bone loss[336] when compared to sham infected animals. Different groups have expressed bone loss as a percentage of root exposed[406]. Probably the most logical expression of bone loss is as a distance in millimetres from the CEJ to ABC[407 336] and this is the technique we followed in our studies.

2.12.5.6. Statistical analysis

Morphometric analysis of bone loss is extremely technique sensitive. Firstly the sample must be perfectly oriented on the microscope table such that the buccal and lingual cusps are superimposed to ensure it is perpendicular to the line of viewing. Secondly the image must be successfully captured. Thirdly, the measurements must be made on a computer screen using anatomical landmarks that must be correctly identified.

Inter-operator and intra-operator reliability is therefore paramount to ensure the accuracy of bone loss measurement. The studies vary from no mention of repetition and blinding[401] to a high degree of care being taken to ensure reliability; 3 separate measurements on different days by 2 blinded examiners analysing randomised samples[336].

In the studies presented in this thesis the majority of the analyses were carried out by a single operator negating the possibility of inter-operator calibration and comparison. Intra-operator reliability was maintained by repeating the measurements for a random selection of samples on two separate occasions. The degree of intra-operator reliability was assessed using Pearson's Correlation Coefficient and is shown in Appendix 7 for each bone loss figure. A coefficient approaching 1 and a level of significance of $p<0.05$ was considered as a high degree of correlation. This measure was used as it allows for correlation between measurements carried out on different occasions on samples that were mounted independently for each measurement. By using this approach it takes into account the possibility that the measurements might be different due to slightly different orientation of the sample at each measurement. For example, if the sample was mounted at a different angle on the occasion of the second measurement all the measurements could be increased or decreased by a similar amount depending on this orientation. This type of correlation takes into account the fact that all corresponding measurement might be increased or decreased by a similar amount whilst still giving a measure of reliability. A degree of blinding was possible by coding of the samples.

2.12.5.7. Limitations of morphometric analysis

This methodology is highly technique sensitive in terms of handling the sample, taking the measurements and analysing these measurements.

The main limitation of this technique is that it uses two dimensional information to assess what is in reality a complex three dimensional problem. Periodontal bone loss is by its nature not linear in fashion. Infra bony defects are often seen both in the clinical and experimental situation. This can be illustrated when comparing the morphometric technique with the new gold standard technique of Micro Computed Tomography[407]. When a sample is oriented as the Morphometric protocol dictates an underestimate of bone loss can occur because it fails to take into account the infrabony element of the defect (Figure 2.16).

Another limitation of the Morphometric technique is in the processing of the samples. All the soft tissue is removed prior to analysis to expose the bone. Consequently, the ability to perform histology and immunohistochemistry is lost. In a Micro CT technique the tissue is fixed and preserved for later analysis.

In these studies we decided to use the morphometric technique to generate large quantities of raw data

2.12.5.8. Conclusions

Morphometric analysis of periodontal bone loss is a simple and valid way of measuring bone loss. It is, however, extremely technique sensitive and requires a high degree of skill and experience to achieve the reliability of other methods. With this in mind it is desirable to generate a large pool of measurement data so that when it is averaged out the inaccuracies due to technique are minimised. It is also important to perform repeated samplings at different time points and with blinded (ideally multiple) examiners to maintain inter and intra-operator reliability.

The major drawback of this technique is its failure to account for the infrabony defects that are a feature of periodontal bone loss. This can lead to significant underestimates of the amount of bone loss. The processing of the samples is also destructive to the soft tissue removing the possibility of carrying out any histological techniques.

2.13. Aims and objectives

2.13.1. Summary of Literature review

Periodontal disease is one of the most prevalent chronic diseases that affects the human population and is a significant drain of health care resources in the UK and worldwide. It can be highly debilitating and its effects are largely irreversible. Although the presence of specific distinct disease entities have been identified the net result is always progressive destruction of the periodontal tissues and ultimately premature loss of teeth if left to progress.

Although multi-factorial in its aetiology the prime driving factor is the microbial plaque biofilm that accumulates on the teeth and in the subgingival niches. Moreover, it is the homeostatic balance between the host and this persistent microbial challenge that determines the nature and progression of the disease process. De-regulation of this homeostasis leads to changes in the biofilm (dysbiosis) and activation of pro-inflammatory pathways in the host in response. Ultimately, this inflammation results in the breakdown of the periodontal tissues.

The presence of the plaque biofilm and the host response to it are the key drivers of the pathogenesis of periodontal disease. More importantly the concept of dysbiosis (deleterious changes in the commensal microbiota) is a key factor in the development of a number of chronic inflammatory diseases including periodontal disease. However, the transition of a normal commensal microbiota into a dysbiotic microbiota is still poorly understood. Specifically, how micro-organisms (e.g. *Porphyromonas gingivalis*) traditionally thought of as pathogenic might influence this transition.

Whilst there is no perfect animal model to investigate every aspect of periodontal disease pathogenesis the rodent model, specifically the oral gavage mouse model, offers a useful tool for investigating the effects of challenging these animals with pathogenic micro-organisms and the effects on the host response. However, the characterisation of the oral commensal microbiota in these mice is poorly understood and therefore the pathogenesis of periodontal disease through dysbiosis has not been investigated in these mice.

It is with all these points in mind that this work set out to investigate the commensal microbiota in the mouse and to look at the effect of a periodontal pathogen on this microbiota, specifically in terms of dysbiosis and the effect on measurable periodontal disease. In addition, the variation and stability of different oral commensal microbiomes in different mice and interactions with specific components of the innate host response through the use of knockout mice were examined.

2.13.2. Hypothesis

Periodontal disease is driven by dysbiosis of the oral commensal microbiota.

2.13.3. Aims and objectives

The specific aims and objectives were as follows:-

1. To define and categorise the oral commensal microbiota of the C3H and Balb/c mouse using culture and non-culture techniques;
2. To investigate the effect of a 'red complex' periodontal pathogen, *Poprhyromonas gingivalis*, on this oral commensal microbiota and its potential to induce dysbiosis in this microbiome and to further investigate the resultant effects on alveolar bone loss;
3. To investigate the influence of the genetic background of the mouse on the oral commensal microbiota and the effects this has on alveolar bone loss;
4. To investigate the transfer of the normal and dysbiotic oral commensal microbiota from specific pathogen free mice into germ free mice and the effects on alveolar bone loss.

Chapter 3

Materials and methods

3. Materials and Methods

3.1. *Porphyromonas gingivalis* culture and Inoculum preparation

Porphyromonas gingivalis W50 was obtained originally from Professor P D Marsh, Porton Down, Salisbury, UK[408] and stored on beads in a glycerol (10-20%) base at -80^{o}C (Microbank Preservation System, Pro-Lab Diagnostics). To resuscitate the micro-organisms, 2 beads were placed from the frozen vial and spread onto blood agar plates supplemented with 5% (v/v) defibrinated horse blood and incubated for 48 hours under anaerobic conditions: 80% Nitrogen, 10% Hydrogen and 10% Carbon Dioxide at 37^{o}C[409] in an anaerobic chamber (Modular Atmospheric Controlled System, Don Whitley Scientific, UK). After 48 hours colonies were picked off the blood agar using a sterile 5µl disposable loop and placed into a sterile Universal tube containing 10ml of Brain Heart Infusion (BHI) (supplied by Oxoid, Thermo Scientific, UK) supplemented with haemin (5µg ml^{-1}). This starter broth culture was then incubated under the same conditions for 24 hours. From this starter culture the culture can be expanded 10 fold (e.g.10ml starter culture into 100ml of BHI and haemin). Purity checking was carried out by noting the characteristic aroma of the broth culture. In addition a sterile disposable 5µl loop of the broth culture was plated onto blood agar and incubated anaerobically. On inspection the plates consisted of a single morphological type of micro-organism which developed black pigmentation over the period of 1 week.

Porphyromonas gingivalis W50 was maintained on blood agar plates containing 5% (v/v) defibrinated blood or grown in Brain Heart Infusion supplemented with haemin (5µg ml^{-1}). Anaerobic culture conditions were maintained for storage and growth at 80% Nitrogen, 10% Hydrogen and 10% Carbon Dioxide at 37^{o}C [409] (Modular Atmospheric Controlled System, D W Scientific, UK). Regular purity checks were carried out by inspection of the blood agar plates for growth of single morphological black pigmenting micro-organisms and characteristic odour.

Inocula for oral gavage of mice were prepared from 24 hour cultures of *P. gingivalis* W50 grown under anaerobic conditions on blood agar plates with added defibrinated horse blood. The bacteria were in the early stationary growth phase. A sterile disposable 5µl loop of the plated micro-organism was placed into 10ml of BHI with haemin and incubated under the same conditions for a further 24 hours. The inoculum was then prepared by suspending 1 x10^9 Colony Forming Units per millilitre (CFU ml^{-1}) as determined by optical density at a wavelength of 600nm (OD_{600}). Previous growth assays were used to relate optical density to the number of CFU ml^{-1}. In short, 1ml of broth culture (BHI + haemin) corresponding to an OD_{600} of 1 was found to be equivalent to 5 x 10^9 CFU so to achieve the desired 1 x10^9 CFU, 200µl of broth culture was therefore required. Alternatively CFU ml^{-1} can be determined by comparison with a standard curve for optical density against time for growth in broth media[202, 410]. The required volume of broth culture was centrifuged at 16,000g for 3 minutes to pellet the bacteria. The bacteria were then suspended in 100µl of filter sterilised 2% (w/v) Carboxymethylcellulose (CMC) (Sigma-Aldrich, International) in Phosphate Buffered Saline (PBS) by vortexing for 10 seconds.

3.2. Mice

All animal procedures performed in these studies were carried out under the Animals (Scientific Procedures) Act 1986 in accordance with United Kingdom Home Office guidelines under the project licence PPL 70/6833 (to M A Curtis) and personal licence number PIL 70/22741 (to M A Payne).

3.2.1. Laboratory mouse phylogenetic tree

All inbred laboratory mice are considered to be a mixture of 4 different subspecies; *Mus musculus musculus*, *Mus musculus domesticus*, *Mus musculus castaneus* and *Mus musculus molissinus*[411]. Recent DNA sequence data using mitochondrial DNA shows that all laboratory mice have a common female wild ancestor of the *Mus musculus domesticus* strain (Figure 3.1). Both mouse strains used in these studies, C3H (Figure 3.2) and Balb/c (Figure 3.3), are aligned along common branches of phylogenetic tree.

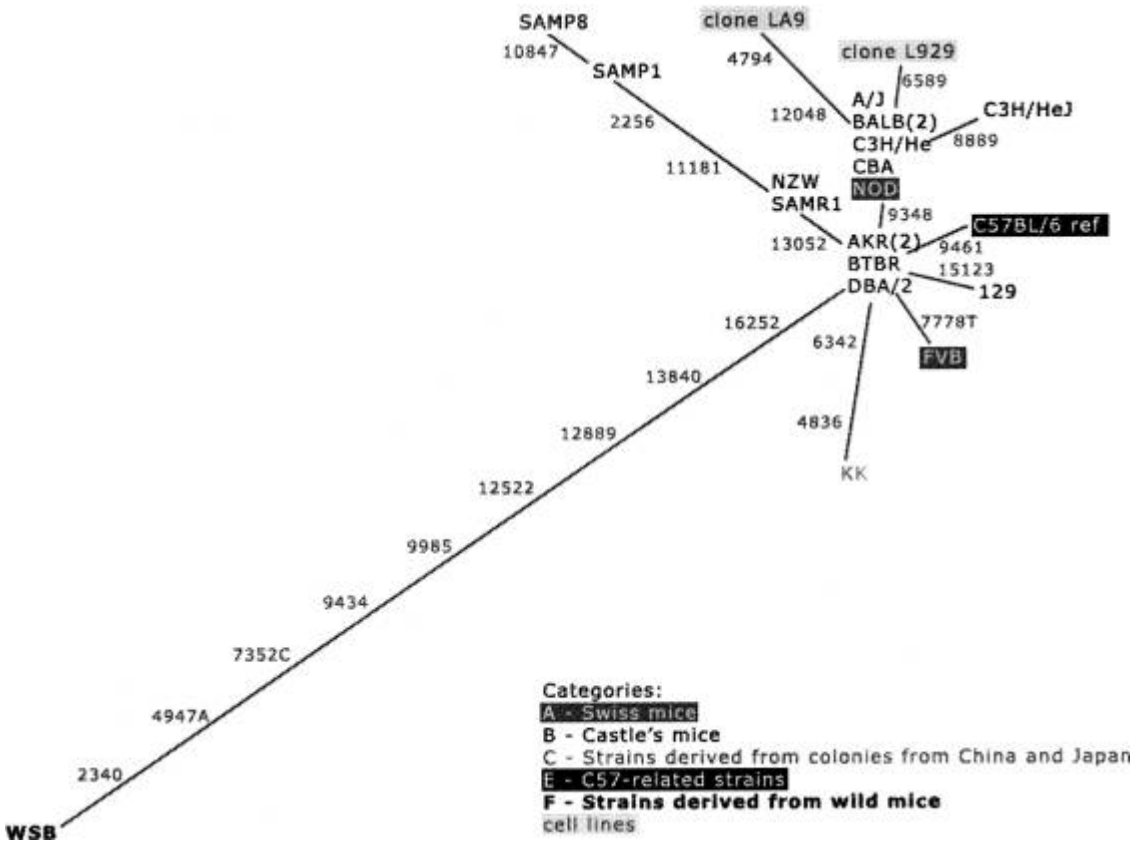


Figure 3.1 Phylogenetic tree showing laboratory mice lineage from a common wild derived inbred strain (WSB) based on micochondrial DNA polymorphisms found in 16 different strains of laboratory mice. Genome Research 2007[411].
http://genome.cshlp.org/content/17/3/293.long

Figure 3.2 C3H/Orl mouse the background in which the colonies of specific pathogen free (SPF) and germ free mice were bred.

Figure 3.3 Balb/c AnNCrl mouse the background in which the CXCR2$^{-/-}$ mice were bred

3.2.2. Establishment of Germ Free (GF) colony

A breeding pair of C3H/Orl germ free mice were purchased from Charles River International and delivered to the germ free facility at the Royal Veterinary College (RVC), University of London. Upon delivery these mice were examined for sterility by aerobic and anaerobic culture of oral swabs and faecal pellets on blood agar supplemented with 5% (v/v) defibrinated horse blood. The animals were housed in isolators in the RVC where periodic checks were made to ensure the germ free conditions were maintained (Figure 3.4). Successive generations provided an established germ free colony in the C3H/Orl strain.

Figure 3.4 Germ Free Isolators similar to the ones used for germ free mice in these studies. Image of a similar facility at the Unit for Laboratory Animal Medicine of the University of Michigan Medical School. http://ulam.med.umich.edu/services/animal/germfree.html

3.2.3. Establishment of a genetically identical Specific Pathogen Free (SPF) colony

Specific pathogen free (SPF) C3H/HeNcrl mice were purchased from Charles River International in 2 separate batches. The oral microbiota was assessed upon delivery by taking oral swabs and growth of cultivable micro-organisms on blood agar plates with 5% (v/v) defibrinated horse blood. Based on culture, 2 distinct microbiota were found in each batch (Table 3.1 and Figure 3.5) delivered from the supplier. Similar variations in the mouse oral microbiota in different batches of the same mice have been found to be the case in other strains of mice[412].

Bacteria were identified by isolating and sub-culturing the colonies from the mixed blood agar plates. Identification was by 16S rDNA sequencing of the isolates and interrogation of NCBI Nucleotide database (see section 3.5 for details) using the Basic Local Alignment Search Tool (BLAST) algorithm for the best homology over the largest section of the gene.

Bacteria identified by 16S Sequencing	SPF Batch 1	SPF Batch 2
Streptococcus sp	1.2×10^7	2.5×10^7
Lactobacillus murinus/animalis	3.8×10^4	2×10^6
Staphylococcus aureus	5.8×10^4	7.4×10^4
Staphylococcus haemolyticus	*ND	8.1×10^4
Staphylococcus sciuri	4.7×10^3	ND
Enterococcus casseliflavus	1.35×10^5	ND
Microbacterium lacticum	1.4×10^4	ND
E. coli/Shigella sp	5.5×10^3	ND
Propionibacterium acnes	3.3×10^5	ND
Bacteroides acidifaciens	1.5×10^4	ND

Table 3.1 Identification of the cultivable oral micro-organisms of C3H/HeNcrl mice bought in 2 different batches from the supplier. Micro-organisms are expressed as $CFUml^{-1}$. Identification was based on 16S rDNA sequencing and BLAST interrogation. Data courtesy of Dr Ahmed Hashim.

Figure 3.5 Histogram showing the comparative differences in oral microbiota in two different batches delivered from the suppliers. Individual colonies were isolated based on morphology from the samples on oral swabs grown for 48 hours under aerobic and anaerobic conditions. These individual colonies were then identified by 16S rDNA sequencing. Each bacterial species is expressed as their proportion in the total cultivable bacteria. Data courtesy of Dr Ahmed Hashim.

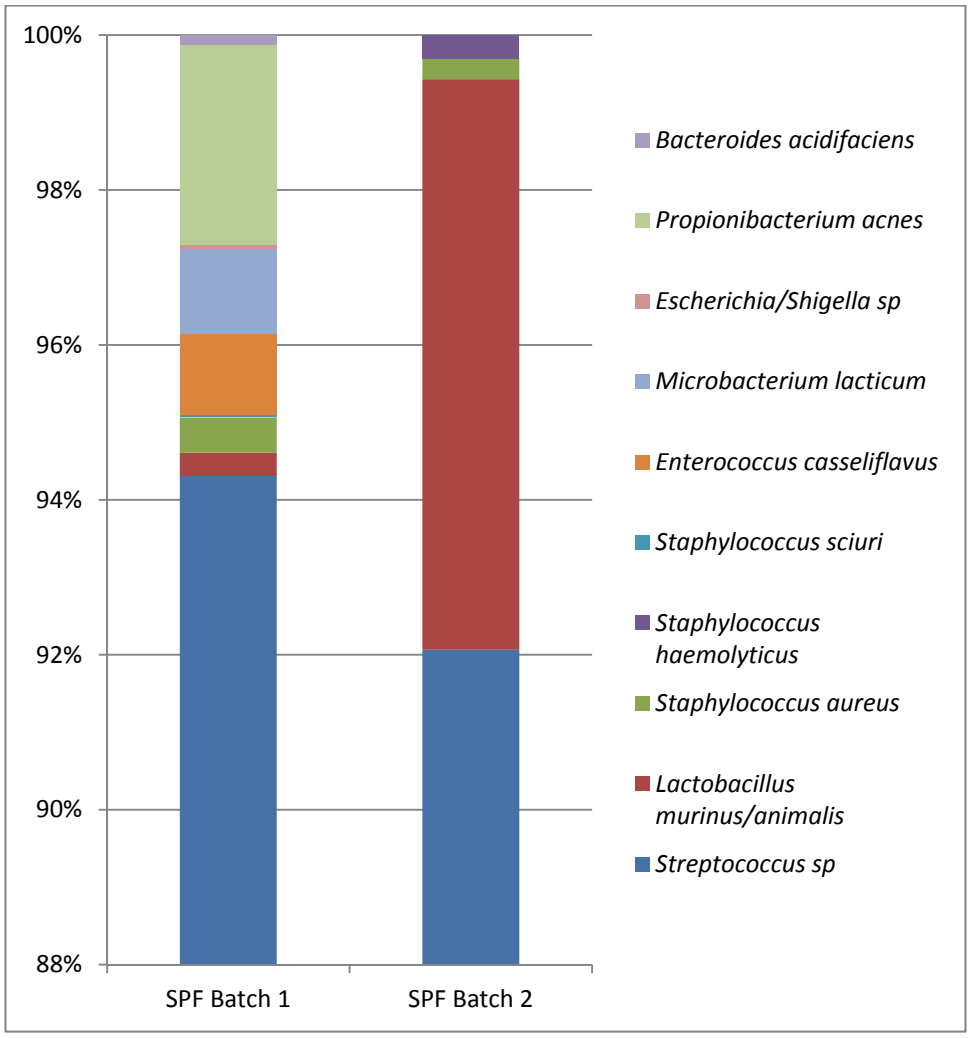


Figure 3.5

Based on the analysis of the commensal oral microbiome of the 2 batches of C3H/HeNcrl mice it was decided to select the simpler of the two microfloras (SPF Batch 2) and use this as our 'normal' microflora for this strain of mouse. This simpler microbiota was made up of 4 main constituents that could be detected reliably by culture methodology. There were genetic differences between our GF colony (C3H/Orl) and our SPF colony (C3H/HeNcrl) so in order to generate an SPF colony genetically identical to the germ free, the C3H/HeNcrl SPF oral microbiome was transferred into the germ free C3H/Orl by co-caging. Co-caging was carried out in a ratio of 1 SPF mouse to 2 GF mice. We found that the transfer of the micro-organisms cultivable by aerobic or anaerobic means on blood agar occurred within 2 weeks such that at this time point the commensal microbiota of both sets of mice was identical (see section 4.1.1.). The previously GF mice (who had received the SPF microbiota) were then used to establish our breeding colony of SPF from which all successive SPF mice were descended. This meant all mice were now the same C3H/Orl genetic background and with a defined (at least on the basis of culture) commensal oral microbiota.

All SPF mice were housed in individually ventilated cages (IVCs) in the Biological Services Unit, Queen Mary University of London (Figure 3.6). All animals, SPF and GF, received identical irradiated extruded feed (5053 PicoLab® Rodent Diet 20, PicoLab, Brentwood MO 63144) and irradiated water *ad libitum*. The mice experienced 12 hour light-dark cycle and were maintained at 25^{o}C.

Figure 3.6 Individually Ventillated Cages (IVCs) for housing SPF mice in the Biological Services Unit of Queen Mary University of London.

Balb/c AnNCrl mice (Charles River Laboratories International) were ordered as required as controls for gene knockout mice and for transfer experiments. Their oral microbiome was examined by culture and non-culture techniques upon delivery (see section 4.7). They were maintained in IVCs and received feed and water as detailed above.

3.2.4. Knockout mice

Additional SPF colonies of genetically modified mice were used in some co-caging experiments. CXCR2 $^{-/-}$ mice in a Balb/c background (see Appendix 1) were homozygous with a targeted mutation in the gene that codes for the receptor (CXCR2) of mouse homologues of Interleukin 8 (IL-8). These homologues include CXCL1 (Groα) and CXCL2 (Groβ). The CXCR2 receptor is a major mediator of neutrophil recruitment[70] and is expressed by the neutrophils. The CXCR2 ligands (CXCL1 and 2) are expressed in the gingival tissues by epithelial cells and are a potent chemo-attractant for neutrophil migration into the gingival tissues. These mice exhibit increased and accelerated periodontal bone loss both spontaneously and following oral gavage with *P. gingivalis*[383-385]. They also have splenomegaly, lymphadenopathy and impaired neutrophil migration.

For oral gavage with *P. gingivalis*, male and female C3H mice were used. These were sex and age-matched to 8-10 weeks at the start of the experimental period and were euthanized with CO_2 6 weeks following the last inoculation (aged 14-16 weeks). Cage numbers ranged from 2 to a maximum of 6 mice per cage. Male litter-mates could be housed in the same cages but males from different litters could not as they show aggression and fighting. Females from different litters could be housed in the same cages. Males and females were kept separate so breeding could not occur.

For co-caging experiments females only were used. Mixing of males from different litters led to excessive aggression and fighting resulting in unacceptable injury to the mice. There were insufficient numbers in each litter to allow co-caging of males, hence only females were used.

3.3. Oral Gavage

This method of oral inoculation of mice to induce periodontal bone loss was pioneered by Baker et al[336, 413] with slight modifications subsequently[206].

In brief, the mice were first given antibiotics to suppress the normal oral flora. This regime consisted of addition of Co-trimoxazole (480mg/5ml) oral suspension (supplied by the Pharmacy Department, Royal London Hospital) added to the irradiated drinking water. It was given at a dose 20ml per litre of water (final concentration of 320mg trimethoprim and 1.6g of sulphamethoxazole per litre of water) for 10 days followed by a 3 day 'wash out' period on normal irradiated drinking water prior to the first inoculation with *P. gingivalis*.

Following preparation of the inocula as detailed above, 50 µl of the bacterial cell suspension were inoculated directly into the stomach using a ball ended feeding needle (Figure 3.7A & B) and 50 µl were inoculated into the oral cavity using a Gilson pipette (Figure 3.8 A & B). Inoculation directly into the stomach allowed the infecting micro-organisms to colonise the gut which then acts as a further reservoir for the infective agent when mice engage in coprophagia. Inoculations were carried out on 3 occasions separated by 48 hours. All inoculations were carried out in a laminar flow cabinet under aseptic conditions (Figure 3.9). Control groups were 'sham' inoculated with the CMC carrier vehicle only. Following the last inoculation the mice were euthanised with CO_2 6 weeks later when the animals were aged 14-16 weeks.

A

B

Figure 3.7 The ball ended feeding needle and syringe (A) used for inoculation of *P. gingivalis* directly into the stomach (B).

A B

Figure 3.8 Oral gavage directly into the stomach with a ball ended feeding needle (A) and into the oral cavity with a Gilson pipette (B).

Figure 3.9 A Laminar flow cabinet used to carry out all oral sampling and inoculations under sterile conditions.

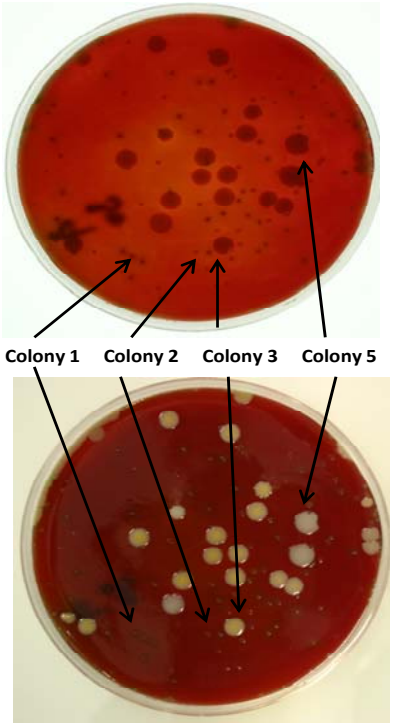
3.4. Oral Sampling

All microbiological sampling was performed in a laminar flow cabinet. Sterile cotton wool swabs were introduced into the mouse oral cavity and moved over the teeth, tongue, cheeks and tonsils for 30 seconds.

For samples that were for processing for culture methodologies, the swabs were immediately placed in sterile reduced John's transport medium (See appendix 2 for details). Samples were processed for culture on blood agar plates within 1 hour of collection. The samples were diluted 1:10, 1:100 and 1:1000 in BHI supplemented with haemin (5µg ml^{-1}) and cultured aerobically and anaerobically for 48 hours. Colony counts were performed at 48 hours (Figure 3.10 A&B). Individual colonies were distinguished based on differing morphologies and a total count was produced for each morphologically different colony and expressed in colony forming units per millilitre (CFU ml^{-1}). Total aerobic and anaerobic counts were then produced by summation of these morphologically different colonies and expressed in CFU ml^{-1}. The individual colonies were isolated and sub cultured for identification. Sequential sampling at different time points allowed examination of the microbial counts of the commensal microbiota over time.

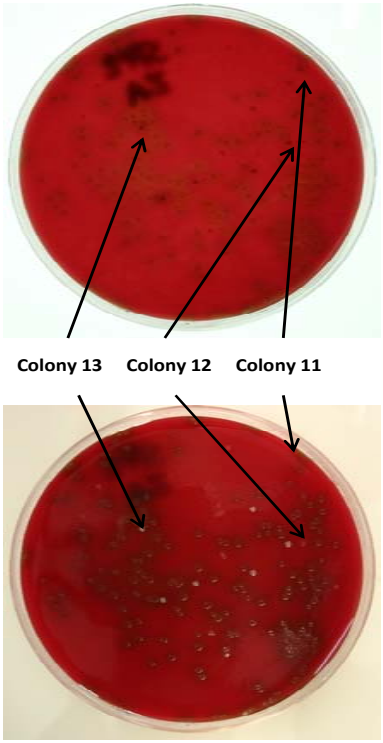
For samples that were processed for non-culture techniques, the swabs were immediately placed into Tris-buffered Ethylenediaminetetraacetic acid (TE) at a concentration of 10mM Tris and 1mM EDTA at pH8. The swabs were then swirled in the TE for 30 seconds before being removed and placed into a dry sterile Eppendorf tube. The supernatant TE was used for processing the DNA. The swabs were stored dry in Eppendorf tubes at -80^{o}C.

Figure 3.10 A Blood agar plates showing typical colony types after 48h aerobic incubation. Individual colonies based on morphological differences were isolated and subcultured for identification by MALDI-TOF mass spectrometry or 16S rRNA gene sequencing.



Colony 1: Small, round, convex, entire, black halo
Colony 2: Small, round, convex, entire, no black halo
Colony 3: Medium, irregular roundish, convex, entire, cream/yellow
Colony 5: Medium, irregular roundish, convex, entire, grey

Figure 3.10 B Blood agar plates showing typical colony types after 48h anaerobic incubation. Individual colonies based on morphological differences were isolated and subcultured for identification by MALDI-TOF mass spectrometry or 16S rRNA gene sequencing.



Colony 11: Small, round, entire, convex, pitted centre, black halo
Colony 12: Small, round, entire, convex, ghostly white
Colony 13: Small, round, entire, convex, white

3.5. Bacterial Identification

Individual colony types were identified based on morphology. These individual colonies were sub-cultured by plating onto fresh blood agar containing 5% (v/v) defibrinated horse blood. Identification of cultivable micro-organisms was carried out on sub-cultured isolates following 48 hour culture of the blood agar plates under aerobic and anaerobic conditions.

3.5.1. Mass spectrometry of cultivable isolates

Some samples were identified by use of matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry[414] supplied by Bruker Corporation. In short, the sample is first desorped by use of an ultra-violet (UV) laser. This produces a hot plume which is then ionized by the laser. This ionized hot plume consists of many different types of molecules of many different sizes. These molecules pass along a vacuum tube before being detected at the other end; their time of flight being dependant on their mass. The resultant 'fingerprint' of detected peak similarities and intensities allows identification when compared to a database of known MALDI-TOF traces.

Sample preparation involved ethanol/formic acid extraction of fresh bacterial isolates grown overnight. 5-10mg of the fresh colony was placed into an Eppendorf tube containing 300µl water and vortexed briefly. 900µl ethanol was added and mixed by inversion. The sample was then centrifuged at 16,000g for 2 minutes and the supernatant discarded, ensuring no residual ethanol remained. 25µl of 70% formic acid was added and vortexed for 5 seconds before 25µl of pure acetonitrile was added. Following further centrifugation at 16,000g for 2 minutes, a 1µl spot of the supernatant was placed onto the target plate and allowed to air dry. This was covered by 1µl of matrix solution (alpha cyano hydroxycinnamic acid in acetonitrile) and allowed to air dry for 10 minutes. Control organisms were fresh (<2 day old) *Escherichia coli* ACTC 25922.

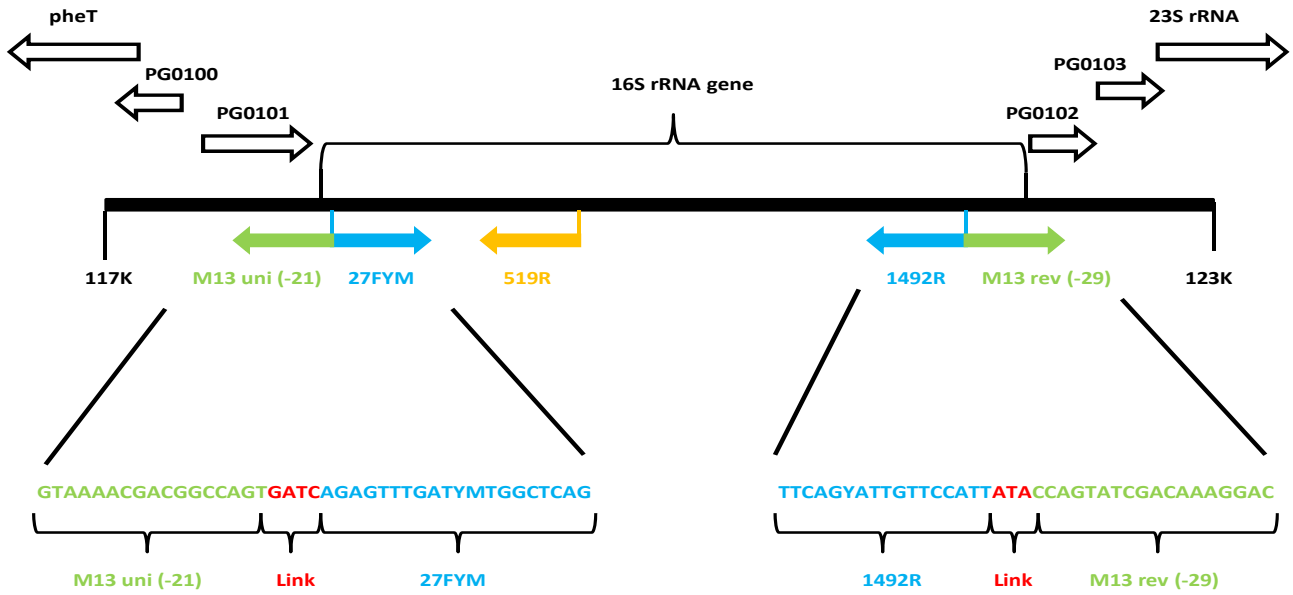
Resultant peaks were compared to a database and presented as a $\log_{10}$ value up to 3. Acceptable values for positive identification are from 2.2 (very good identification) to 2.4 (excellent identification). See Appendix 3 for identification tables.

3.5.2. Sequencing of cultivable isolates

16S rDNA sequencing[415] was used to identify cultivable isolates. DNA from fresh isolates was extracted using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Briefly, harvested cells were treated with lysozyme (45mg/ml) to digest the cell walls. They were then lysed with Proteinase K (20mg ml^{-1}) and lysis solution before mixing with ethanol and binding to silica membrane in a micro-centrifuge tube. Following double washing the DNA was then eluted and collected.

Amplification of bacterial 16S gene [414] was then performed using the following primers:-BLIZF2 which consisted of 5'-GTAAAACGACGGCCAGTGATCAGAGTTTGATYMTGGCTCAG-3'(forward) and BLIZR2 which consisted of 5'- CAGGAAACAGCTATGACCATATTACCTTGTTAYGACTT-3' (reverse) both at 100µM concentration. These primers were designed as 16S primers forward and reverse, 27FYM and 1492R respectively (blue sequences) linked by a linkage sequence (red sequences) to forward and reverse M13 primers (green sequences) M13 uni (-21) and M13 rev (-29) respectively. The resultant amplicon (normally ~1500 bp) has integral domains for the M13 primers (Figure 3.11). Amplification was carried out using Extensor master mix and the following thermocycler parameters; initial denaturation 95°C for 5 minutes, 25 cycles of denaturation at 95°C for 1.5 minutes, annealing at 47°C for 1.5 minutes, extension at 72°C for 3 minutes, final extension at 72°C for 10 minutes and cooling to 10°C. Samples were run on 1% agarose gels in TBE with ethidium bromide to assess quality of amplicons. Amplicons were purified with MinElute PCR purification kit (QIAGEN) and eluted into 50µl TE.

Figure 3.11 One region of the 16S rRNA bacterial gene amplified by primers 27FYM (forward) and 1492R (reverse) for *P. gingivalis* W83 (blue sequences). These primers were linked to the M13 forward and reverse primers (green sequences). Diagram based on NCBI genome sequence of *P. gingivalis* W83 open arrows represent flanking regions of gene between 117Kbp and 123Kbp.



Determination of concentration of the purified PCR product was performed using NanoDrop and samples were standardized to 150ng in 15µl for sequencing. Sequencing was carried out by MWG Biotech International using the following M13 primers: - M13 uni (-21) 5'- TGTAAAACGACGGCCAGT -3' (forward) and M13 rev (-29) 5'- CAGGAAACAGCTATGACC -3' (reverse).

From the forward and reverse sequences a consensus was generated (usually ~1400bp) using BioEdit which was then interrogated against the National Center for Biotechnology Information (NCBI) nucleotide sequence database using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Controls were known strains of *P. gingivalis* W50 presenting as 99% sequence identity over 100% of the region. Identification was based on the 'best hit' on the database which showed greatest homology over the largest part of the sequence to the database reference (Appendix 6).

3.5.3. Construction of 16S rDNA library

Identification of bacteria described so far has been reliant on culture techniques. The bacteria have all been grown on non selective media under standard aerobic or anaerobic conditions. This includes the sequence data which has been derived from isolates that have required culture to grow. From studies in humans and the Human Oral Micrbiome Project and Database (HOMD)[112] we know that the complete oral microbiome of humans includes a significant percentage of non-culturable bacteria (~68%). Similar studies in mice using next generation sequencing techniques have shown the mouse oral microbiome to be more diverse than would be suggested by culture methods alone[416]. Hence there was a need to utilize non-cultural techniques, specifically conventional Sanger sequencing of cloned 16S rDNA libraries, to generate information on both the cultivable and non-cultivable microbiota of a number of different mice.

Oral swabs from the mice were swirled in TE (10mM Tris, 1mM EDTA, pH8) for 30 seconds then removed to eliminate retention of any micro-organisms within the swab itself. The supernatant was then centrifuged at 16,000g for 1 minute to pellet the cells before further washing in TE. The resultant pellet was processed for DNA extraction using the GenElute Bacterial Genomic DNA kit as above.

Extracted DNA was amplified for bacterial 16S rRNA gene using the following primers[417]:- 27FYM 3'- AGAGTTTGATYMTGGCTCAG-5' (forward) and 1492R 5'-TACCTTGTTAYGACTT-3' (reverse). This typically produced amplicons of around ~1500bp. The thermocycler programme was identical to that used for amplifying DNA from isolates as in section 3.16.2. Amplicons were purified with QIAQuick PCR product clean up kit (QIAGEN) and eluted into 50µl TE. Quality of purified amplicons was examined on 1% agarose gels in TBE with ethidium bromide.

Cloning was carried out using TOPO TA Cloning Kits (Invitrogen International). This technique allows direct insertion of the Taq-polymerase-amplified PCR products into a plasmid vector for sequencing. The system utilizes the single deoxyadenosine (A) residues that are added to the 3' ends of the 16S PCR products by the Taq during this amplification. The plasmid vector (pCR4-TOPO) has single overhanging deoxy-thymidine (T) residues allowing efficient ligation with the PCR product. In addition, this vector allows positive selection of recombinants by its ability to disrupt the lethal *E. coli* gene *ccd*B. When unopposed by the poly-peptide, the gene *ccd*A, *ccd*B encodes a protein that is responsible for gyrase-mediated double stranded DNA breakage and induction of the SOS pathway. In the vector the *ccd*B gene is fused to the C-terminus of the LacZα fragment so ligation with a PCR product disrupts expression of the *lac*Zα-*ccd*B gene permitting growth of positive recombinants upon transformation in TOPO10 cells. Cells containing non-recombinant vector are killed upon plating as the *ccd*B gene is not disrupted (Figure 3.12).

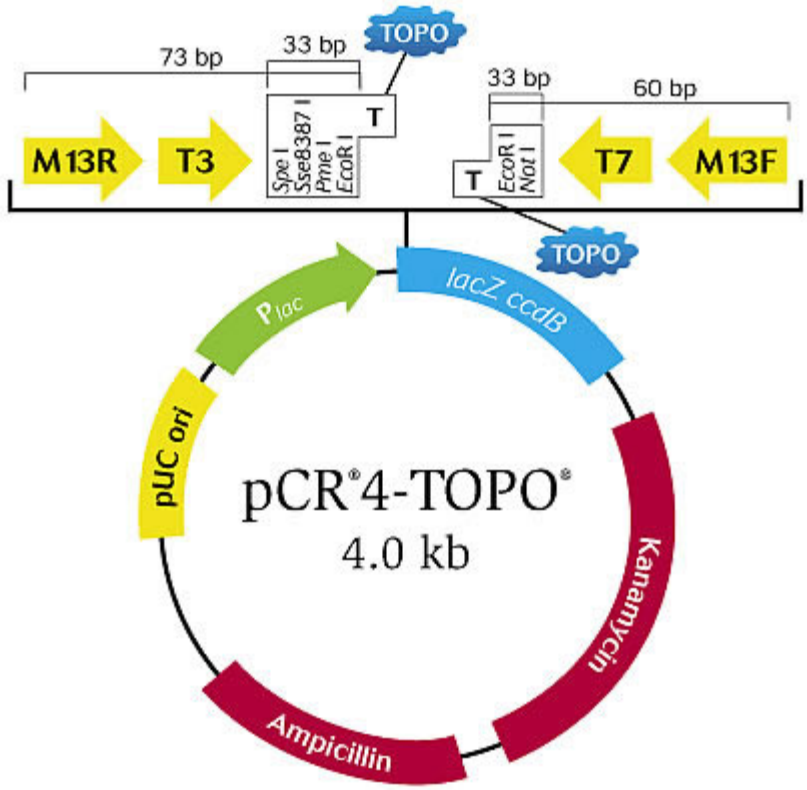


Figure 3.12 pCR4-TOPO vector used for cloning, the overhanging T residues allow the 16S rRNA PCR product to ligate and there are flanking M13 primers to allow amplification of the recombinant plasmids. Diagram courtesy of Invitrogen
http://products.invitrogen.com/ivgn/product/K457501

The cloning reaction was carried out using fresh PCR products following 16S amplification. A total reaction volume of 6µl was required made up of 1µl TOPO vector, 1µl salt solution (1.2M NaCl and 0.06M $MgCl_2$), 0.5-4µl PCR product and water. To optimize the system, it was discovered early that the maximum 4µl of PCR product was required because of the relatively low yield of DNA after the initial DNA extraction. This mixture was incubated at room temperature for 30 minutes before transferring to ice. 2µl of the ligation mix was added to each tube of Transform One Shot Mach1-T1 Competent Cells (*E. coli*) and incubated on ice for 10 minutes. They were then heat shocked for 30 seconds at 42^{o}C before adding 250µl of Super Optimal Growth Media (S.O.C – see appendix 4 for details) and agitation at 200rpm and 37^{o}C for 1 hour. The resultant broth culture was plated onto Luria Bertani (LB) agar plates containing Kanamycin (50µg/ml) and incubated overnight aerobically at 37^{o}C.

Approximately 100 random transformants from each cloning reaction were taken from all plates using a sterile wooden cocktail stick. These were 'patched' into individual wells of a 96 well plate with each well containing 100µl of LB and kanamycin. The plates were incubated at 37^{o}C for 6 hours until turbidity could be seen. These inserts of the recombinant plasmids in these clones were then directly re-amplified with flanking region of M13 primer sites derived from the cloning vector.

The M13 primers were as follows: - 5'-GTAAAACGACGGCCAG-3' (forward) and 5'-CAGGAAACAGCTATGAC-3' (reverse). The thermocycler parameters were initial denaturation at 95^{o}C for 5 minutes, 25 cycles of denaturation at 95^{o}C for 0.5 minutes, annealing at 55^{o}C for 0.5 minutes and extension at 72^{o}C for 2 minutes, a final extension at 72^{o}C for 4 minutes and cool to 10^{o}C. Quality of M13 amplicons was assessed on 1% agarose gels in TBE with ethidium bromide.

Final clean up and sequencing of ≤600bp of the sequence was carried out by MWG International using 519R primers as follows: - 5'- GTATTACCGCGGCKGCTG -3'. Resultant sequences were assessed for quality by visual inspection of chromatograms using BioEdit and using internal quality control systems of MWG. Sequences that were satisfactory were analysed by BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal RNA Database Project website (http://rdp.cme.msu.edu/). Sequences were then aligned using ClustalW and trees constructed using the MEGAN 4 programme[418-420] (Appendix 5). Identification was performed at the genus level and also as a 'best-hit' approach the BLAST database (Appendix 6).

3.6. Detection of *Porphyromonas gingivalis*

Colonisation of the mouse oral cavity by *P. gingivalis* was determined by means of anaerobic culture methods, with examination of plates incubated for times ranging from 48 hours to 2 weeks for the presence of 'black pigmenting' organisms. However, this micro-organism was not detected by this method at any time in mouse oral swabs.

Immuno-fluorescence microscopy was also used to detect *P. gingivalis* in oral swabs. Detection was possible using monoclonal antibody 1B5 (MAb 1B5). This monoclonal antibody recognises an epitope on the glycosylated monomeric cysteine protease RgpA as well as a cell surface polysaccharide. Treating with MAb 1b5 is followed by a Texas Red-labelled anti-mouse secondary antibody[421]. Counter staining with 4', 6-diamidino-2-phenylindole (DAPI) allowed comparison to all bacterial cells present in the oral swab.

In addition, PCR was performed on the samples using a 16S rRNA gene species-specific PCR oligonucleotide primers: 5'- AGGCAGCTTGCCATACTGCG-3' (forward), 5'-ACTGTTAGCAACTACCGATGT-3' (reverse)[422]. Genomic DNA extracted from parental *P. gingivalis* served as positive control with no template DNA as negative control. A Techne Thermocycler was used with the following programme; 2 minutes at 94^{o}C, 35 cycles of 30 seconds at 94^{o}C, 30 seconds at 50^{o}C, 1 minute at 72^{o}C before final extension for 2 minutes at 72^{o}c and hold at 10^{o}C. The resultant product was visualized following electrophoresis on a gel of 1% agarose in TBE with ethidium bromide.

3.7. Alveolar bone loss

The morphometric technique for measuring alveolar bone loss was pioneered in the 1980s in Buffalo, New York[401]. It involves preparation of samples by removing the flesh from the jaws. The jaw bones are then positioned and carefully oriented under a microscope where direct measurements of bone loss can be made.

Sample preparation involved the dissecting out of the mandibles and maxillae from the euthanized mice. Enzymatic digestion of the soft tissue using a proprietary detergent containing alkaline bacterial pronase (Terg-A-Zyme)[404] then followed with the samples incubated at 50^{o}C in a 4% solution of Terg-A-Zyme for 3 days. This was the optimal time; too short and the flesh was not fully removed and too long and the teeth became lose and were spontaneously lost due to dissolving of the periodontal ligament.

To measure alveolar bone loss 25x magnification on a Stemi SV11 dissecting microscope (Zeiss, Germany) and image capture facility (Nikon) was used. The images were analysed using Image J software (National Institute of Health, USA, http://rsb.info.nih.gov/ij) with pixel measurements being converted by hand into millimetres with reference to the image of a 1mm graticule scale.

Positioning of the sample on the table of the microscope is vital to ensure the accuracy of the measurements. The original protocol[401] calls for the sample to be oriented such that the buccal and lingual cusps are superimposed i.e. the sample is perpendicular to the line of sight.

There is wide variation in the literature on which point and even which jaw the bone loss measurements are carried out. The original technique[401] measured 12 points on the buccal surfaces of all teeth in both jaws. Other key workers[336] in this area measured 14 points on the buccal surfaces of maxillary molars only. This latter method has been the standard to date and used in the most recent studies in this area[385].

There are 2 major issues with this technique. Firstly, the majority of the bone loss occurs on the lingual/palatal side so is missed by just measuring the buccal surfaces.

Secondly, the buccal basal and surrounding bone is thicker and more extensive consequently it is much more likely to mask any infra bony defects leading to an overall underestimation of alveolar bone levels (see section 2.12.4).

Measurements in these experiments were made using a hybrid technique. Both mandibles and maxillae were assessed with measurements being taken at 3 points (mesio-, mid- and disto-) for each tooth and on both buccal and lingual/palatal sides (18 measurements per jaw). This produces a data set on average 60% greater than the conventional methods.

The measurements for each mouse were used to produce a mean value for that individual animal with these individual means being used to generate a mean value with standard deviation for each experimental group.

3.8. Histological analysis

Decalcified sections of the jaw bones were prepared by fixing the dissected tissue with 4% paraformaldehyde solution. The fixed tissues were then decalcified using EDTA-Cacodylate solution (EDTA, Sodium Cacodylate, Sodium Hydroxide) over a period of 4-6 weeks with gentle rocking. The decalcifying solution was changed every 3-5 days. The decalcified tissue was dehydrated through a series of increasing concentrations of ethanol (70-100%) and xylene before embedding in paraffin wax.

Two consecutive 10µm sections were cut every 30 µm in a transverse manner (bucco-lingually) across the teeth. These sections were mounted, de-waxed prior to Tartrate Resistant Acid Phosphatase (TRAP) staining which was performed using Acid Phosphatase, Leukocyte Kit (Sigma-Aldrich International). De-waxed sections (reverse of xylene-ethanol concentrations) were brought to a lower pH by immersing in acetate solution. The slides were then immersed in warmed (37^{o}C) TRAP stain (de-ionised water, diazotized Fast Garnet GBC Solution, Napthol AS-BI phosphate solution, acetate solution and tartrate solution) and incubated for 1 hour at 37^{o}C. Controls were treated with the same solution without the tartrate to highlight all acid phosphatase-positive

cells. Following rinsing the slides were counterstained with Hematoxylin Solution No 3 for 2 minutes and washed with alkaline tap water. Microscopic examination was performed of the alveolar bone to assess presence of TRAP-positive multinucleate cells which by definition are osteoclasts.

3.9. Statistics

From pilot data we found that the mean bone levels for an SPF mouse was 0.391+/- 0.018mm and for a *P. gingivalis* challenged mouse was 0.508 +/- 0.029mm. Analysis of these data showed it to be normally distributed (Wilks-Shapiro Test).

Power calculations were performed with a sensitivity of detecting a difference in bone level of 1.5% at a significance of p=0.05 with alpha error level of 5% and beta error level of 80%. This showed that n=6 animals per group were required.

Bone levels were compared using one way analysis of variance (ANOVA) and unpaired Student's t test between 2 comparison groups. Significance was expressed at the $p<0.05$ level. This is comparable with previous studies[198].

The majority of these studies involved one examiner so absolute blinding and inter-operator calibrations was not completely possible. However, when bone loss analysis was performed samples were coded and analysed blind. In addition, random samples were measured on two separate occasions and the results compared by Pearson's Correlation Coefficient (See Appendix 7). Reliability was considered satisfactory if the coefficient (r) approached 1 at a statistically significant level ($p<0.05$).

Similarly, for the purpose of calculating sample sizes for microbiological testing we assumed normal distribution among the data set. Pilot data showed the numbers (CFU/ml) of the 2 of the commonest micro-organisms was 2.48×10^5 +/- 5.95×10^4 and 8.2×10^4 +/- 1.59×10^4. Assuming a 10% variation in these values with a similar power of 80% and a significance of $p<0.05$, a sample size of n=4 is required.

Again, only partial blinding was possible due to the nature of collecting and processing the samples and these studies being carried out by a single examiner. Plates were coded and microbial counts performed blind. In addition, periodically, random samples were recounted two separate occasions (within the same day to maintain the 48hour incubation period).

Chapter 4

Results

4. Results

4.1. Defining the oral commensal microbiota

4.1.1. The commensal microbiota of the C3H/Orl mouse

The primary aim was to establish two genetically identical colonies of mice – one Specific Pathogen Free (SPF) and the other Germ Free (GF) (See Section3.2.2. and 3.2.3.). Firstly, the chosen 'normal' commensal microbiota was selected from two different batches of C3H/HeNCrl (Section 3.2.3. Table 3.1). Secondly, it was necessary that this selected commensal microbiota was transmitted fully from the C3H/HeNCrl SPF mice into the C3H/Orl mice, thus having a known commensal microbiota in an SPF C3H/Orl colony and a genetically equivalent C3H/Orl GF colony. The transmission of this commensal microbiota was examined by culture and non-culture techniques.

4.1.2. Culture Techniques

Oral swabs were taken from female SPF mice and cultured under aerobic and anaerobic conditions for 48 hours. Individual colonies were then sub-cultured before identification by MALDI-TOF mass spectrometry and 16S rDNA sequencing. Female GF animals, with no cultivable micro-organisms, were housed in the same cages as the SPF mice in a ratio of 2:1 and oral swabs taken at days 1, 5, 7 and 14. These swabs were processed in the same manner to facilitate identification of the cultivable microbiota.

The major cultivable micro-organisms under aerobic conditions were *Lactobacillus sp*, *Enterococcus sp*, *Streptococcus sp* and *Escherichia/Shigella sp* (Figure 4.1). Typically at a species level the most commonly found micro-organisms were *Lactobacillus murinus* and *Lactobacillus animalis*, *Enterococcus faecalis* and *Enterococcus asburiae*, *Streptococcus sanguinis*, *Streptococcus salivarius*, *Streptococcus gordonii* and *Streptococcus pneumoniae*. From the sequence length obtained with this methodology (<1500bp) it was not possible to distinguish between *Escherichia sp* and *Shigella sp*; typically there was similar homology (~99%) over the same query regions for multiple strains of these *Enterobacteriaceae* (Figure 4.3). To overcome this more sequence data

would be required over a greater length of the genome or biochemical testing performed.

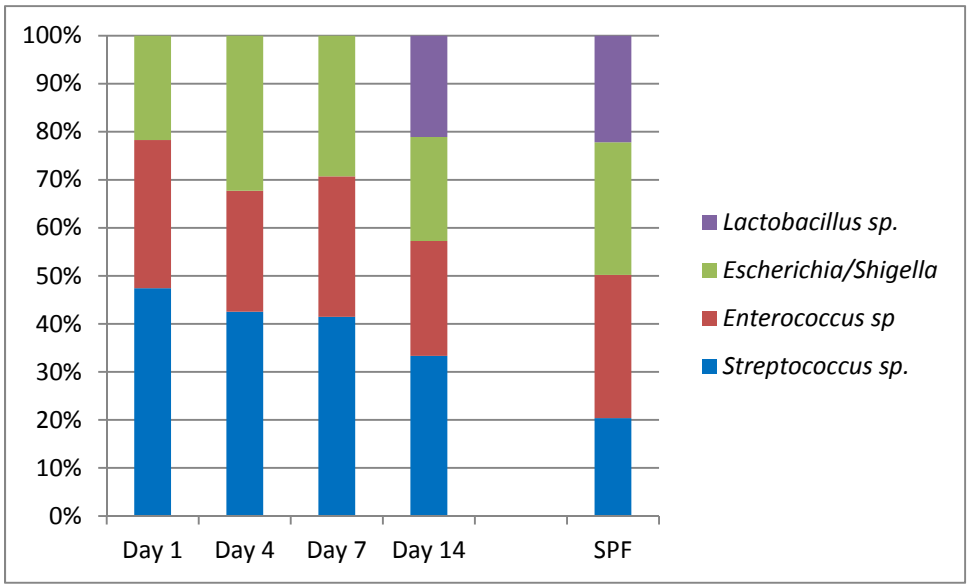


Figure 4.1 Female Germ Free C3H/Orl mice were co-caged with female C3H/HeNCrl SPF mice in a ratio of 2:1. Microbiological sampling at a number of time points showed that there was complete transmission of the aerobic commensal microbiota of the SPF mice into the GF mice by day 14. Each bacterial species is expressed as a percentage of the total bacterial present. Analysis from 16S partial ribosomal DNA sequencing.

The major cultivable micro-organisms under anaerobic conditions were *Propionibacterium sp*, *Staphylococcus sp*, *Streptococcus sp* and *Escherichia/Shigella sp*. The most commonly found micro-organisms at a species level were *Propionbacterium acnes*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus hominis* and *Staphylococcus epidermis*, *Streptococcus salivarius, Streptococcus sanguinis* and *Streptococcus gordonii*. Again distinguishing between *Escherichia sp* and *Shigella sp* was not possible because the percentage homology (≥99%) is very close to the species level.

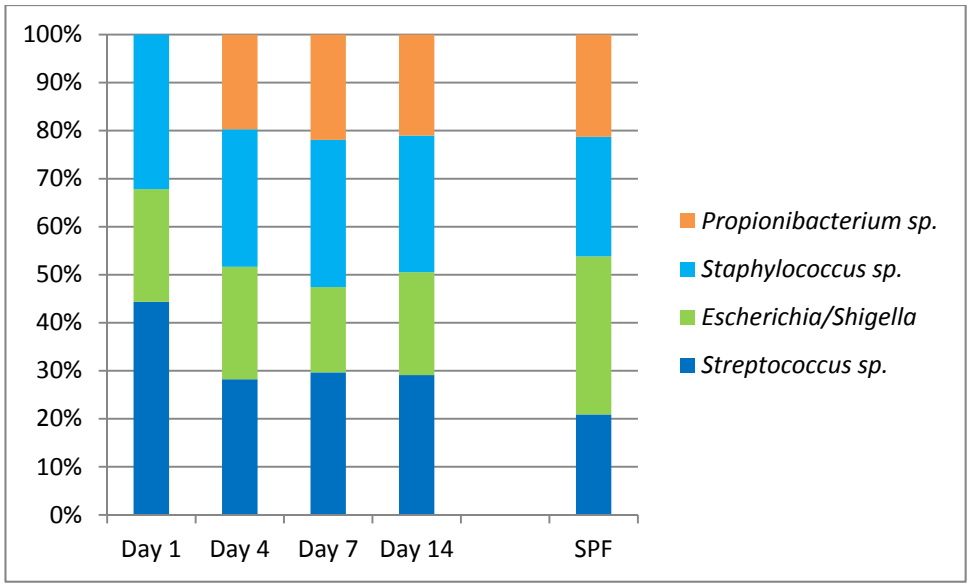


Figure 4.2 Female Germ Free C3H/Orl mice were co-caged with C3H/HeNCrl SPF mice in a ratio of 2:1. Microbiological sampling at a number of time points showed that there was complete transmission of the anaerobic commensal microbiota of the SPF mice into the GF mice by day 14. Each bacterial species is expressed as a percentage of the total bacteria present. Analysis from 16S partial ribosomal DNA sequencing.

There was complete transmission of the major cultivable commensal microbiota from the SPF animals into the GF within 14 days. This occurred for both aerobically cultured micro-organisms (Figure 4.1) and those cultured anaerobically (Figure 4.2). Interestingly, the transfer of *Lactobacillus sp* did not occur in the same way as the other micro-organisms; it did not appear until later in the transmission time line. It may be that this particular micro-organism requires specific environmental conditions to establish and that these are present once the biofilm begins to mature. Typically early colonisers tend to be *Streptococcus sp* and *Actinomyces sp* in humans so it may be a similar situation in the mouse where the colonization by *Streptococcus sp* is required to allow colonization by *Lactobacillus sp*. It is also worth remembering that there are a number of uncultivable micro-organisms that are present that may assist this *Lactobacillus sp* colonisation.

Figure 4.3 Distance tree of results based on neighbor joining analysis for sample Aero 13 (highlighted yellow) showing the close relationship to various *Escherichia sp* and *Shigella sp*. Sample Aero 13 is within 0.2% of 3 *Escherichia sp* and3 *Shigella sp* (red highlighted area).

4.1.3. Non-culture techniques

To further define this commensal microbiota non-cultural techniques were used. A conventional Sanger sequencing approach was taken to the characterisation of a cloned 16S library (Section 3.5.3.). In short, DNA was extracted from oral swabs and the 16S gene was amplified by PCR. Following cloning into *E coli* random transformants were selected for re-amplification of the flanking M13 regions and sequencing. Analysis consisted of BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal Database Project website. Sequences were then aligned using ClustalW and trees constructed from distance matrices using the Jukes-Cantor correction by the neighbour-joining method in the MEGAN software (Appendix 5). Identification was performed at the genus level.

The major bacterial species detected by this method were transmitted from the SPF mice into the previously germ free mice by Day 14 (Figure 4.4). These previously germ free mice are now termed conventionalised (CNV).

Using this 16S sequencing approach there were 3 major bacterial species belonging to the phyla Firmicutes that were consistently detected; *Lactobacillus sp*, *Streptococcus sp* and *Gemella sp*. *Gemella* are Gram-positive cocci that occur in pairs or clusters that are facultative anaerobes[423]. *Gemella palacticanis* forms small pin head colonies on blood agar after 72 hours, which is consistent with the morphology of some of the colonies we have identified. Interestingly *Gemella sp* was a novel finding, the presence of which had not been suggested by culture methodology to this point.

A possible explanation for this is that identification by culture means relies on distinguishing different colonies on a mixed agar plate based on morphological differences. In retrospect *Gemella sp* is morphologically similar to both *Streptococcus sp* and *Lactobacillus sp* so could easily have been overlooked in the initial selection and sub-culture from the mixed agar plates. This is supported by later analyses by culture means in which *Gemella sp* was isolated and identified (section 4.5.1.).

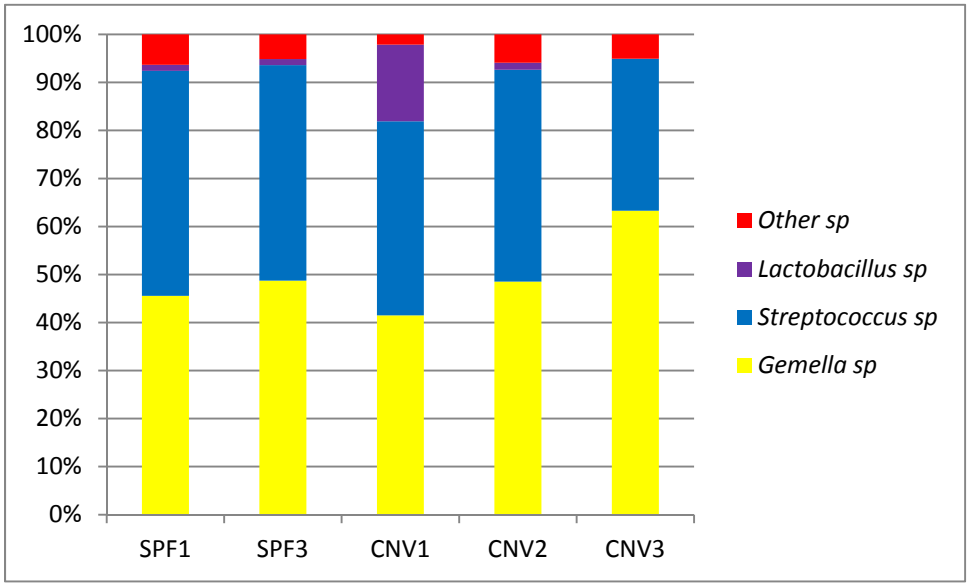


Figure 4.4 Transmission of the major bacterial species from SPF mice to previously GF (CNV) mice at Day 14. Each bacterial species is expressed as a percentage of the number of clones sequenced (typically 80-94). Each column represent sequences based on pooled DNA from either SPF or CNV mice in a cage (typically 2-6 mice). All mice were female.

More detailed analysis of the major microbial species present was performed based on a 'best hit' approach using the BLAST database. This gives identification for each bacteria based on the greatest degree of homology of the sequence with the database over the largest part of the sequence. This approach showed that the *Gemella sp* was exclusively a *Gemella palacticanis*-like micro-organism and the *Lactobacillus sp* was largely a *Lactobacillus animalis*-like micro-organism. However, there was greater diversity in the *Streptococcus sp* present (Figure 4.5). This analysis showed that there were 2 different strains of *Streptococcus sp* that made up the majority of this species; a *Streptococcus alactolyticus*-like and a *Streptococcus sanguinis*-like bacterium. In addition there were *Streptococcus intermedius*, *parasanguinis*, *equi*, *salivarius* and *peroris* – like organisms present in very low numbers.

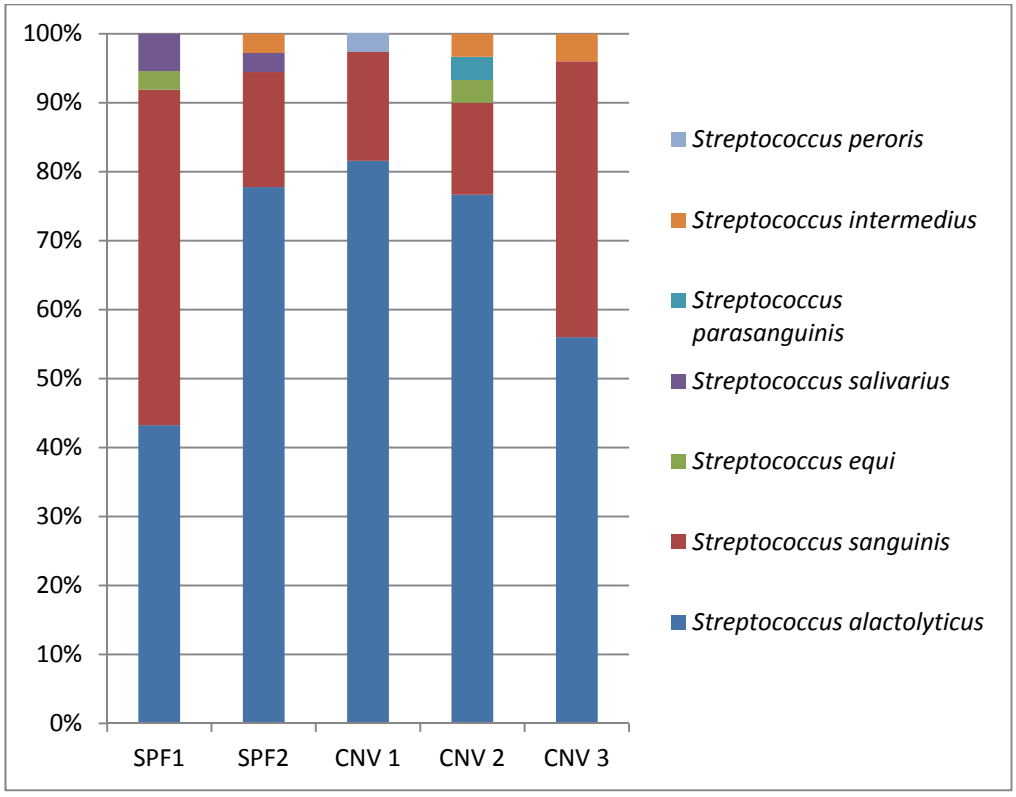


Figure 4.5 Diversity within the *Streptococcus sp* in both SPF and co-caged CNV mice. Identification based on a 'best hit' approach using the NCBI BLAST database. Each strain is expressed as a percentage of the total sequences for *Streptococcus sp*.

Further analysis of the group of micro-organisms termed ‘other’ was performed using a similar approach (Figure 4.6). This diverse range of micro-organisms were largely present in single clones, the most abundant being *Paenbacillus castaneae* which was detected in the SPF mice in 5 and 4 clones out of 5 clones for each sample. This ‘other’ group is highly diverse and represents a group of micro-organisms that are only present in very low numbers in the commensal microbiota. To elucidate these further would require much more sequence data which was not possible using these techniques. A next generation sequencing approach would be required to generate the number of sequences to further characterise these micro-organisms.

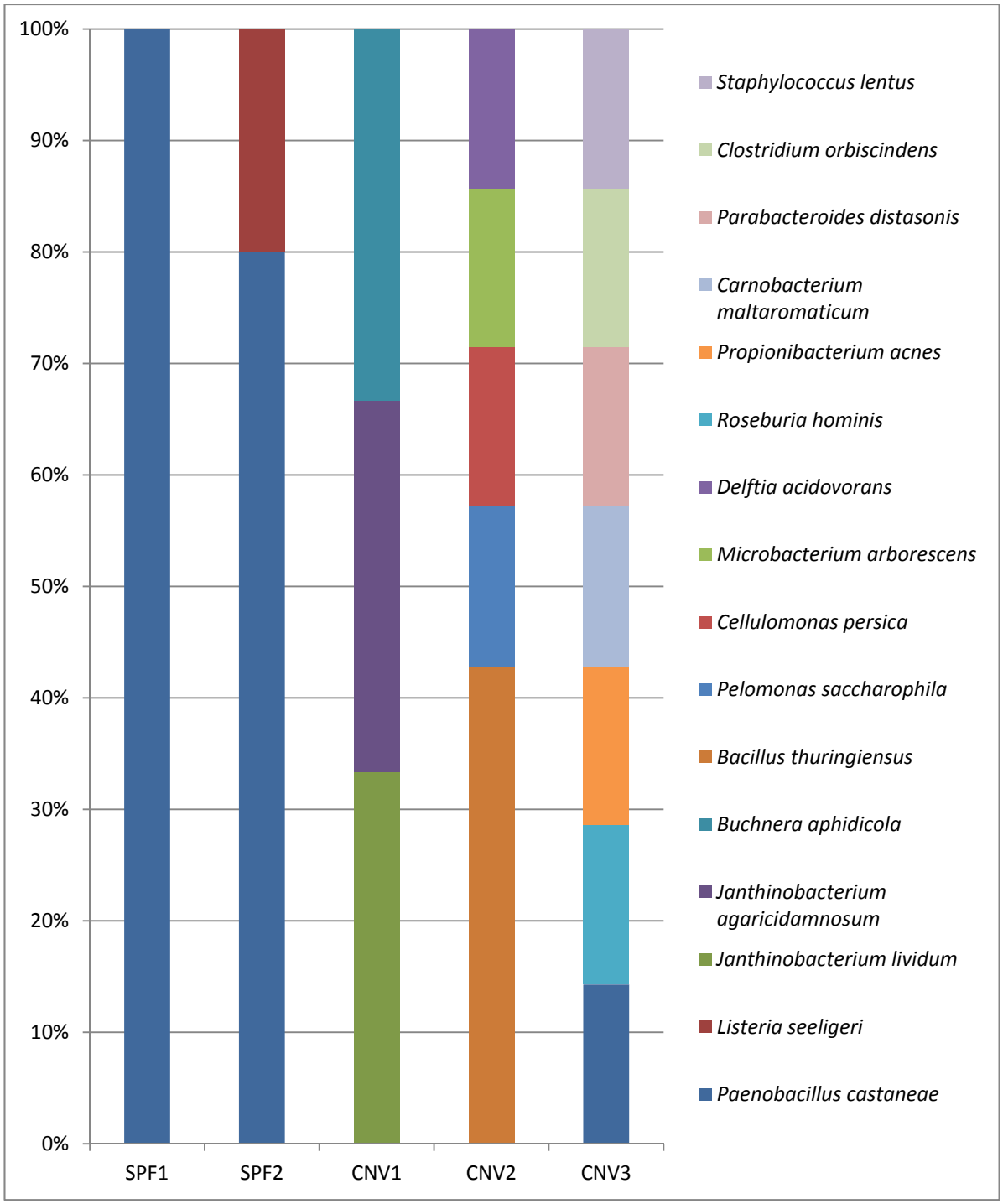


Figure 4.6 Bacterial strains within the 'other' group in both the SPF and CNV mice. Identification based on a 'best hit' approach using NCBI BLAST database. Each bacterial strain expressed as its percentage of the total sequences for the 'other' group which are typically 3-7 clones.

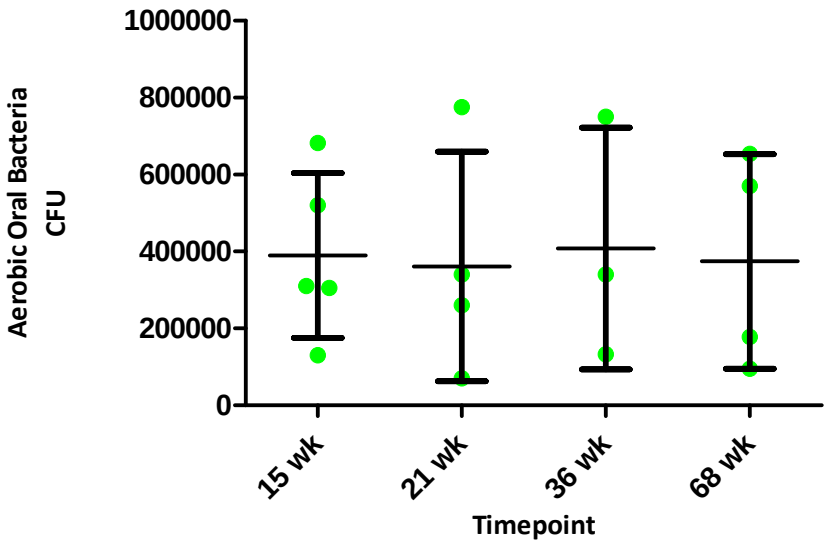
4.2. Effect of aging on the commensal microbiota

The transmission experiment in 4.1 above was performed to establish a known commensal microbiota in a genetic background of mouse (C3H/Orl) which was identical to our germ free mice (also C3H/Orl). Having selected the commensal microbiota it was important to assess the stability of this microbiota over time in mice of differing ages. As the SPF mouse colony developed it was possible to perform oral sampling of a number of mice of different ages. In addition, it was possible to assess the alveolar bone loss in these mice at different ages.

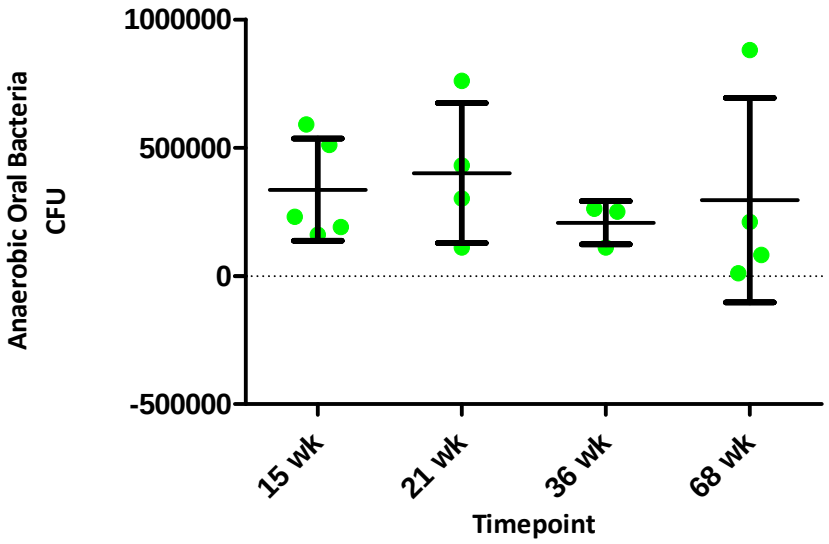
4.2.1. Stability of the commensal microbiota using culture techniques

Oral swabs of mice of differing ages were taken and assessment of the numbers of micro-organisms in commensal microbiota assessed. In short, oral swabs were introduced into the oral cavity of the mouse and all oral surfaces sampled for 30 seconds. These swabs were then placed in reduced John's transport medium (Appendix 2) before serial dilutions were performed on the supernatant. These were plated onto blood agar and incubated for 48 hours under aerobic and anaerobic conditions. After this period of incubation estimates of the counts of each colony type were made based on distinction on morphological differences.

There were no significant differences in the total counts of aerobic and anaerobic micro-organisms detected by culture between mice of different ages (Figures 4.7 A&B).



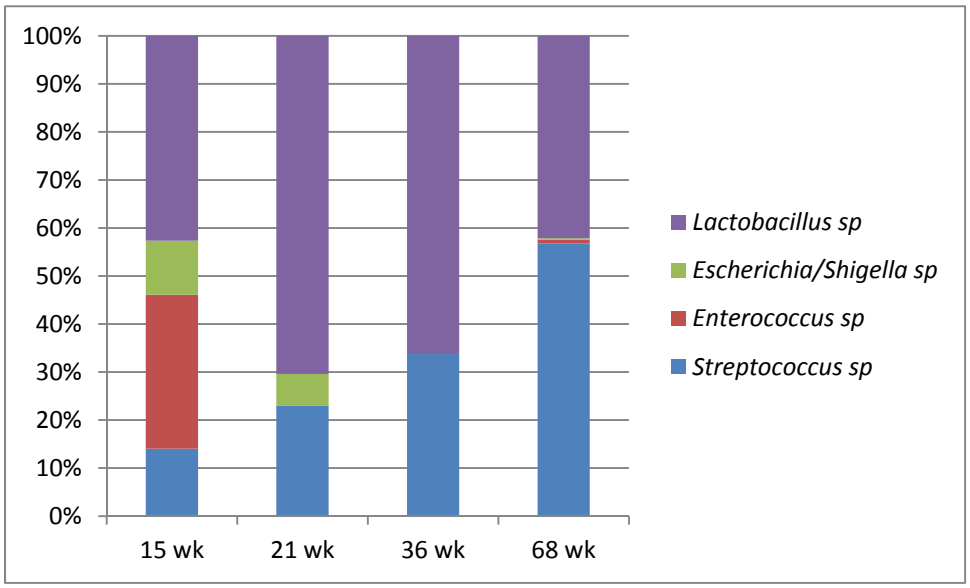
A



B

Figure 4.7 Total microbial counts for micro-organisms cultured aerobically (A) and anaerobically (B) for SPF C3H/Orl mice of different ages. The totals are expressed as total colony forming units (CFU). There were no statistically significant differences between the mice at the ages tested. Each green dot represents the total cultivable oral bacteria for an individual animal, n=3 to n=5 for each time point. Horizontal bars represent the mean values +/- SD.

The composition of the commensal microbiota of mice of differing ages was also determined by culture techniques. Isolates of 48 hour cultures were identified by MALDI-TOF mass spectrometry or by 16S rDNA sequencing. In general, the composition of the commensal microbiota is comparable at the different ages (Figures 4.8 A&B). There was an apparent loss of certain colonies at different ages but this may have been due to the insensitivity of this sampling methodology rather than representing an actual change in composition. In addition, there were the same issues as mentioned in section 4.1.2. when it came to distinguishing colonies based on morphological differences. The non-cultural data (section 4.1.3.) suggest the C3H mouse oral commensal microbiota is stable.



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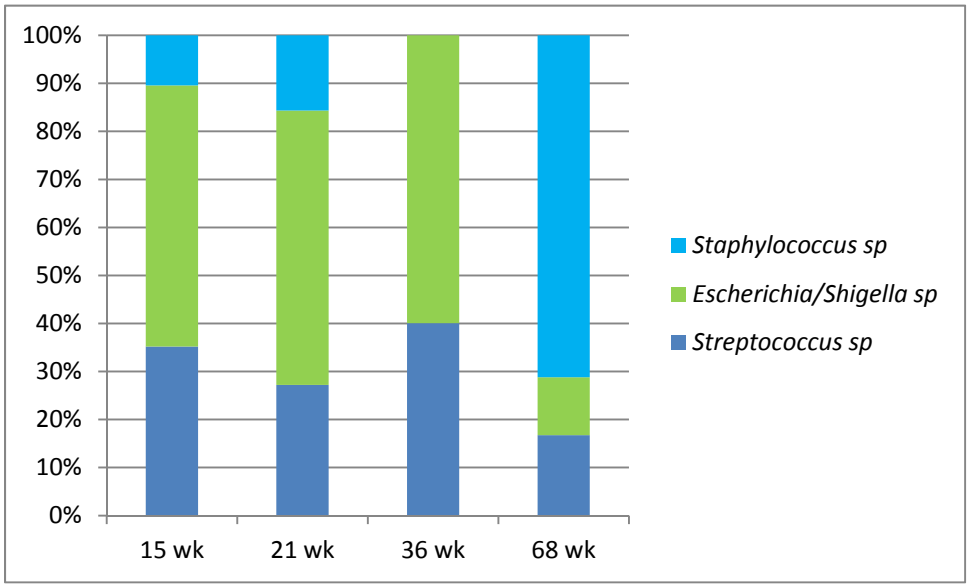


B

Figure 4.8 The composition of the commensal microbiota detected by aerobic (A) and anaerobic (B) culture methods of C3H/Orl mice at different ages. The total for each micro-organism is expressed as an average for each age group then presented as a percentage of the total cultivable commensal microbiota either aerobically or anaerobically. There were 3 to 5 mice in each group. Analysis by 16S partial ribosomal DNA sequencing.

4.2.2. Stability of the commensal microbiota using non-culture techniques

To further investigate the composition of the commensal microbiota with increasing age the DNA from oral swabs was extracted and the 16S gene amplified. Amplicons were then cloned into *E. coli* and a random selection of transformants were sequenced to create a library of sequences. Analysis consisted of BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal Database Project website. Sequences were then aligned using ClustalW and trees constructed from distance matrices using the Jukes-Cantor correction by the neighbour-joining method in the MEGAN software (Appendix 5). Identification was performed at the genus level.

C3H/Orl mice of different ages had a similar composition of the major species in the commensal microbiota (Figure 4.9). 3 major species were detected namely, *Gemella sp*, *Streptococcus sp* and *Lactobacillus sp*. More detailed analysis based on a 'best hit' approach showed that there were only individual strains present within each species; *Gemella palacticanis*-like, *Lactobacillus animalis*-like and *Streptococcus alactolyticus*-like strains.

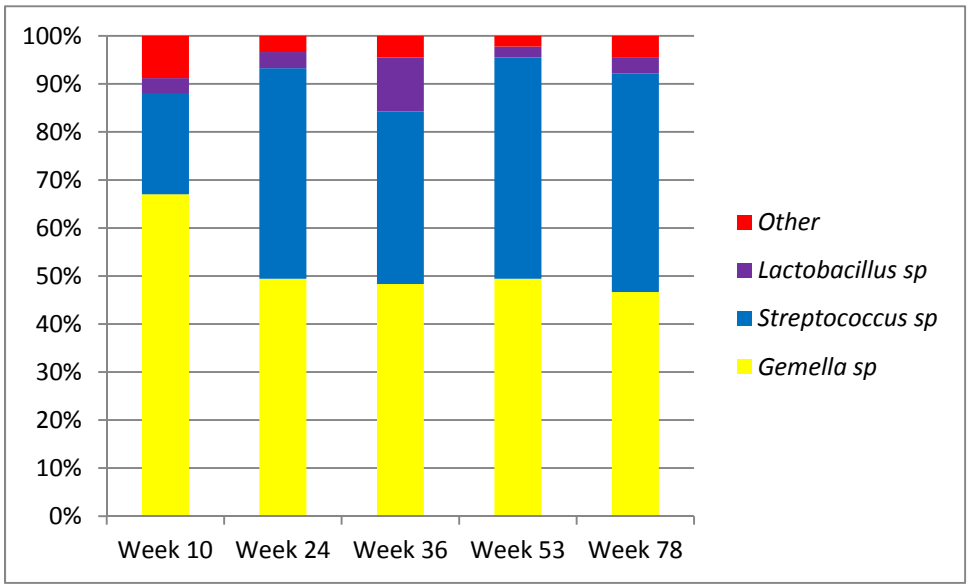


Figure 4.9 The composition of the commensal microbiota in C3H/Orl mice of different ages based on cloning and sequence analysis. Each micro-organism species is expressed as a percentage of the total number of sequences for each age group.

4.2.3. Effect of aging on alveolar bone loss in C3H/Orl mice

Following microbiological sampling of the mice at different ages they were euthanized by CO_2 and measurements of alveolar bone loss made by morphometric means. The results were consistent with other studies in different strains of mice; alveolar bone loss increases with increasing age (Figure 4.10). Bone loss was initially gradually then increased in a more accelerated manner after 9 months of age.

Despite not having a commensal microbiota, germ free (GF) mice also developed alveolar bone loss with increasing age. At each age, the bone loss in the SPF mouse was statistically significantly greater than in the GF mouse ($p<0.003$)

(Intra-operator reliability see Appendix 7.1)

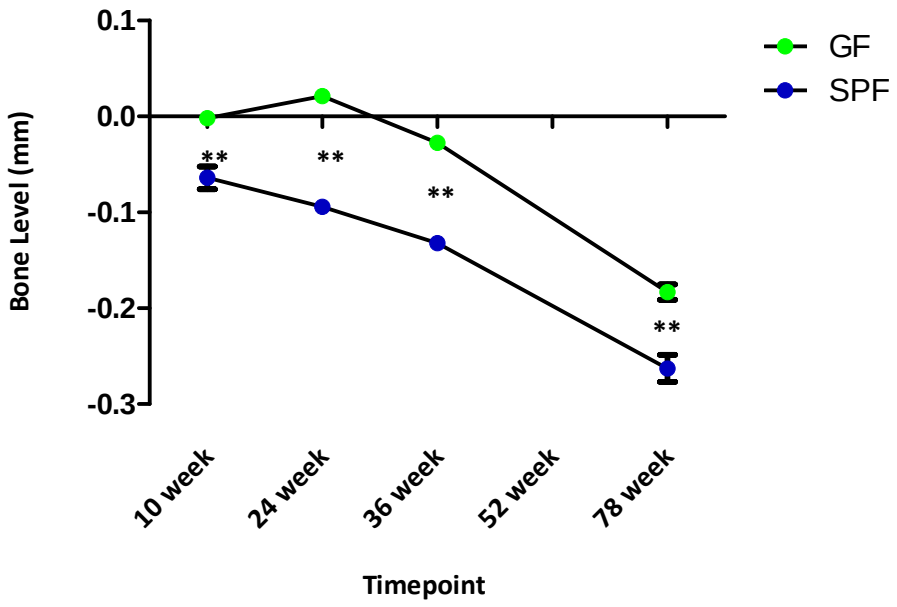


Figure 4.10 Alveolar bone loss in Specific Pathogen Free (SPF) C3H/Orl mice and Germ Free (GF) C3H/Orl mice at different ages. Bone level is relative to a baseline of zero for a 10 week GF mouse. Negative values indicate bone loss relative to this baseline. ** denotes significance at $p<0.003$ between SPF and GF mice of the same age. (Intra-operator reliability see Appendix 7.1)

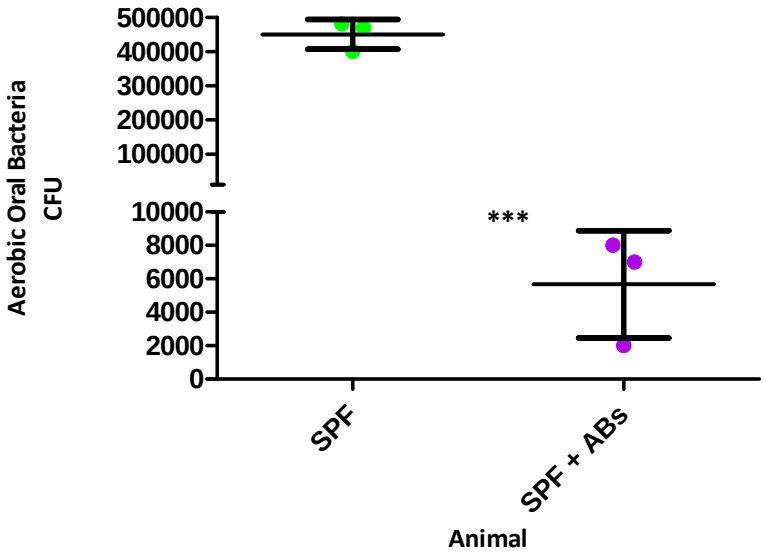
4.3. Effect of antibiotics on the commensal microbiota

The standard oral gavage model of experimental periodontitis[336] required suppression of the oral commensal microbiota with antibiotics prior to oral challenge. To test the effect of the antibiotic regime on the commensal oral microbiota, we used the culture and non-culture techniques as described above.

4.3.1. Effect of antibiotics on microbial counts

The antibiotic regime required 10 days of co-trimoxazole to be administered in the drinking water *ad libitum* then a 3 day 'wash out' period where the mice were administered sterile water. Oral swabs of the mice were taken at the end of the 'wash out' period and assessment of the effect on the numbers of micro-organisms in commensal microbiota made. In short, oral swabs were introduced into the oral cavity of the mouse and all oral surfaces sampled for 30 seconds. These swabs were then placed in reduced John's transport medium (Appendix 2) before serial dilutions were performed on the supernatant. These were plated onto blood agar and incubated for 48 hours under aerobic and anaerobic conditions. After this period of incubation estimates of the counts of each colony were made based on distinction on morphological differences.

Unsurprisingly, the administration of antibiotics significantly decreased the total aerobic (Figure 4.11. A) and anaerobic (Figure 4.11. B) microbial counts.



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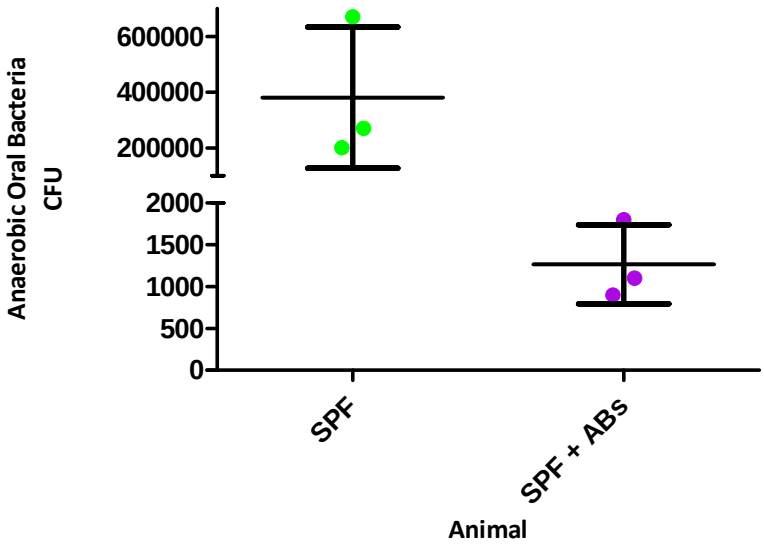

B

Figure 4.11 Total microbial counts of micro-organisms cultured aerobically (A) and anaerobically (B). Cultures were taken from oral swabs following 10 days of co-trimoxazole and a 3 day 'wash out' period. *** denotes significance at $p<0.0001$. Horizontal lines represent mean values +/- SD.

4.3.2. Effect of antibiotics on the composition of the commensal microbiota using culture techniques

Following aerobic and anaerobic cultures for 48 hours individual colonies were isolated and sub-cultured. From these the DNA was extracted and identification made using 16S rDNA sequencing or MALDI-TOF mass spectrometry.

The effect of co-trimoxazole antibiotic on the composition of the oral commensal microbiota was also significant. Pre-treatment there were 3 predominant species of micro-organism detectable following culture under aerobic conditions; *Streptococcus sp*, *Lactobacillus sp* and *Escherichia/Shigella sp* (Figure 4.12. NB *Escherichia/Shigella sp* is a narrow band at the top of the SPF bar on the far left). Following antibiotic administration only the *streptococcus sp* remained detectable by aerobic culture. There was a similar effect in the change in composition of the micro-organisms in the oral commensal microbiota that were detectable by anaerobic culture means; pre-treatment *Streptococcus sp*, *Escherichia/Shigella sp* and *Staphylococcus sp* predominate (Figure 4.12. NB *Staphylococcus sp* is a narrow band at the top of 3rd bar from the left).

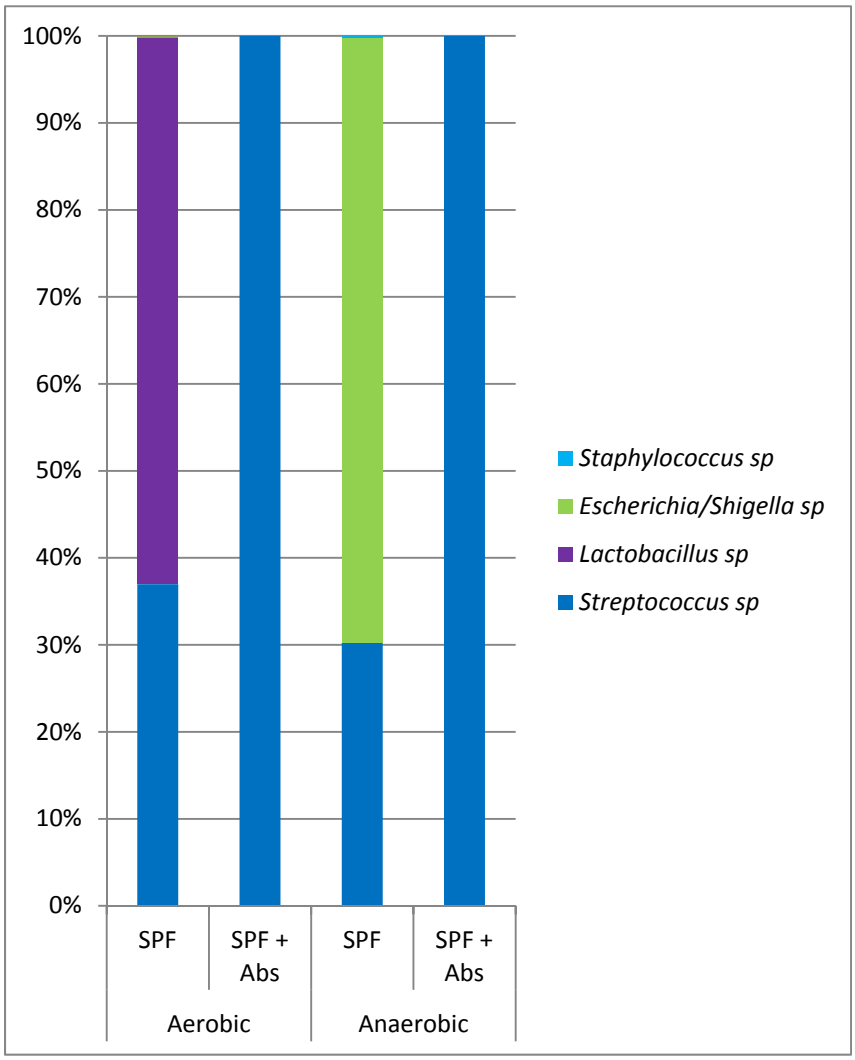

Figure 4.12 Compositional shifts in the commensal microbiota detectable by aerobic and anaerobic culture brought about by the administration of Co-trimoxazole antibiotics. The average count for each micro-organism were calculated and expressed as a percentage of the total micro-organisms present in the commensal microbiota. Analysis by 16S partial ribosomal DNA sequencing.

4.3.3. Effect of antibiotics on the composition of the commensal microbiota using non-culture techniques

Analysis of the oral swabs was also performed at the same time point by non-culture dependent means. In short, DNA was extracted from the oral swabs and the 16S gene amplified by PCR. The amplicons were cloned into *E coli* and random transformants were selected for sequence analysis. Analysis consisted of BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal Database Project website. Sequences were then aligned using ClustalW and trees constructed from distance matrices using the Jukes-Cantor correction by the neighbour-joining method in the MEGAN programme (Appendix 5). Identification was performed at the genus level.

The administration of antibiotics eliminated *Gemella sp* from the commensal microbiota (Figure 4.13) with a concurrent increase in *Streptococcus sp* and *Lactobacillus sp*. *Cellulomonas sp* was the only additional component of the commensal microbiota that was detected and this was exclusively *Cellulomonas persica* when analysis was performed at the species level using a 'best hit' approach on the NCBI BLAST databases as previously described (section 3.5.3.). This was not inconsistent with the data presented throughout this chapter as *Cellulomonas sp* is commonly detected but at low numbers (1-3 clones) such that it is included in the 'other' group of micro-organisms. It was included in these data as it was present in more significant numbers (7-15 clones) in these samples. This may be due to it being over represented in these particular samples or a function of the random selection of transformants.

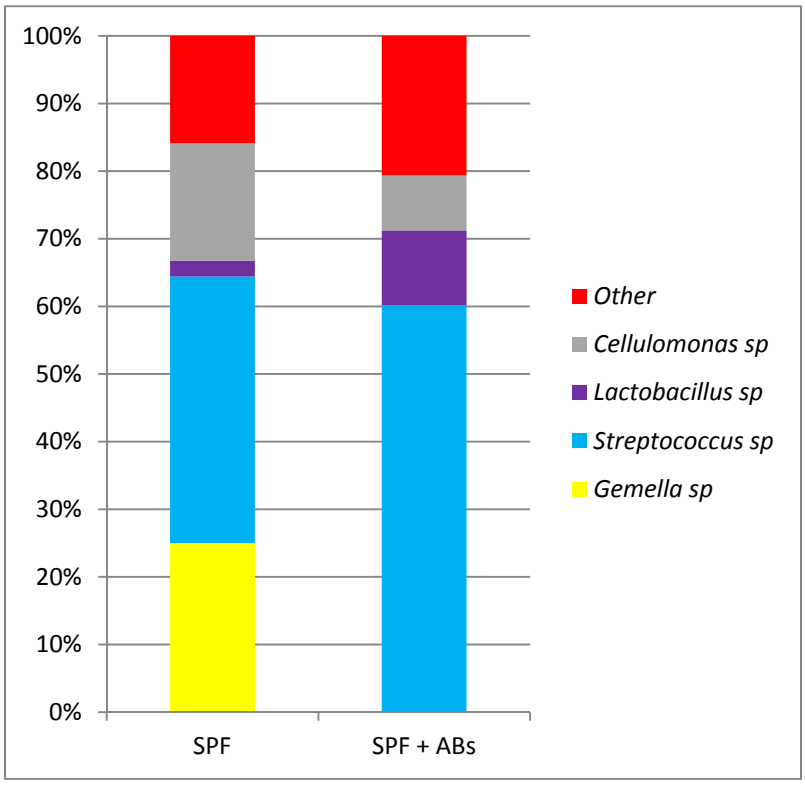

Figure 4.13 The effect of co-trimoxazole antibiotics on the major species of the commensal microbiota detectable by non-culture methodology. 16S rDNA sequence analysis was based on random selection of transformants and each bacterial species is expressed as a percentage of the total number of clones (82-94).

4.4. *Porphyromonas gingivalis* challenge of specific pathogen free (SPF) and germ free (GF) mice

Establishment of 2 genetically identical colonies, one SPF and one GF in C3H/Orl mice was vital to allow the exploration of the pathogenesis of periodontal disease by way of the oral gavage model of experimental periodontitis[324, 336, 413].

In short, the SPF mice were pre-treated with the antibiotic co-trimoxazole for 10 days followed by a 3 day 'wash out' period. These mice were divided into 2 groups; one group receiving *P. gingivalis* in a carboxymethylcellulose carrier vehicle (test group) and the other receiving only the carrier vehicle (control group). Inoculation with *P. gingivalis* (or sham) took place on 3 separate occasions 48 hours apart. The commensal microbiota was monitored before, during and after inoculation by means of cotton wool oral swabs. These swabs were processed and cultured aerobically and anaerobically for 48 hours on blood agar plates. Colony counts were then performed and individual colonies subcultured for identification. The micro-organisms were identified following isolation of 48 hour aerobic and anaerobic cultures by MALDI-TOF Mass Spectrometry and 16S rDNA Sequencing.

The GF mice were received in the Biological Services Unit at the start of the experiment and the lack of a commensal oral microbiota was confirmed by oral swabs as detailed previously (section 3.4.). These mice were divided into 2 groups; a test group and a control group as detailed above. The 2 groups were housed in individually ventilated cages (IVCs) and received irradiated food, water and bedding to maintain as aseptic an environment as practicable. In addition, all inoculation procedures (for both SPF and GF groups) were carried out under aseptic conditions in a laminar flow cabinet.

After 6 weeks all the animals were sacrificed and bone loss was assessed by morphological technique. In short, the jaws were de-fleshed and oriented under a dissecting microscope perpendicular to the line of sight. The image was digitally captured and analysed using Image J software to take linear measurements between alveolar bone crest (ABC) and cement-enamel junction (CEJ) at 6 points around each molar tooth. These data were used to develop a mean bone loss for each animal and from that the mean values of bone loss for each group were determined.

4.4.1. Results of *P. gingivalis* challenge of SPF mice from experiment 1

4.4.1.1. Effect of *P. gingivalis* challenge on alveolar bone Loss

Inoculation of SPF mice (those with a commensal microbiota) with *P. gingivalis* led to statistically significantly ($p=0.0033$) increased periodontal bone loss when compared to sham inoculated SPF controls (Figure 4.14 & 4.15). Similar inoculation of *P. gingivalis* into GF mice failed to induce significant differences in periodontal bone loss ($p=0.7970$). When comparing sham inoculated SPF controls with GF controls the SPF mice have significantly ($p<0.0001$) greater bone loss than those housed under germ free conditions.

(Intra-operator reliability see Appendix 7.2)

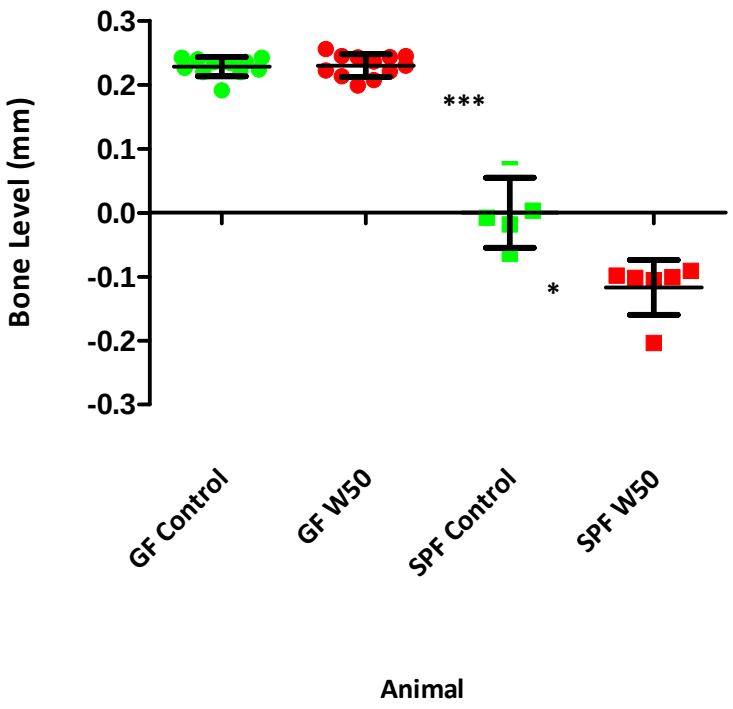

Figure 4.14 Periodontal bone loss expressed as means for each animal with horizontal lines representing the mean value +/- Standard Deviation for each test group. The baseline bone level is that of wild type SPF mice i.e. sham inoculated with all bone loss measured relative to this. Mice were aged 14-16 weeks at sacrifice. Bone loss is expressed as negative change in bone relative to this. * denotes significance at $p<0.05$ & *** at $p<0.0001$. (Intra-operator reliability see Appendix 7.2)

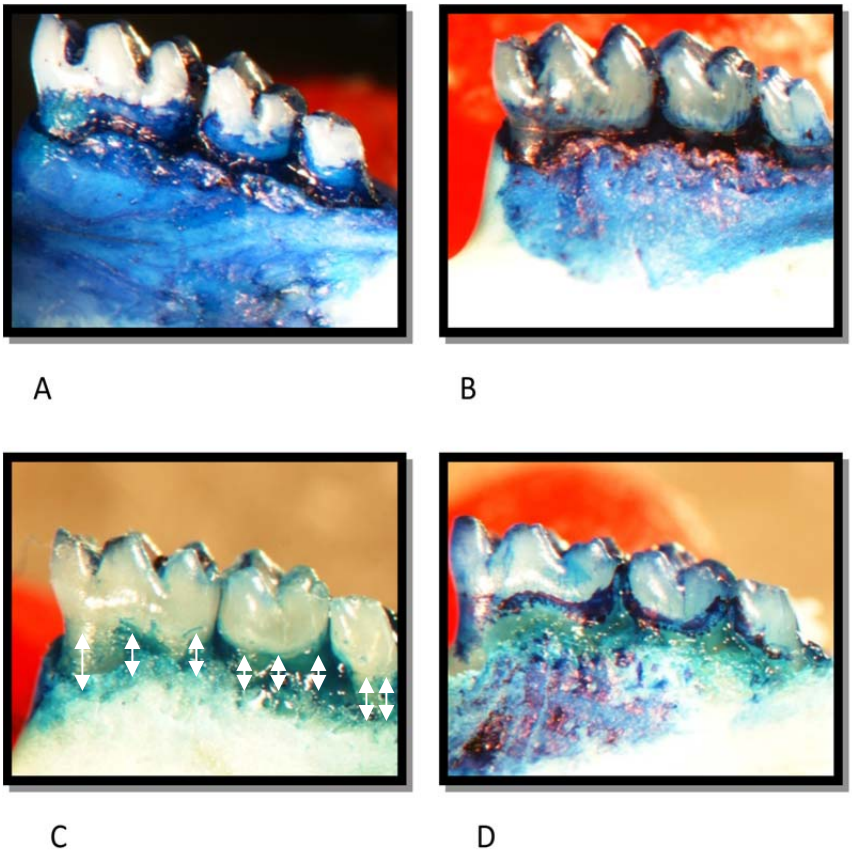

Figure 4.15 Images captured at 25x magnification of the mandibles of Sham inoculated (A) and *P. gingivalis* inoculated (B) Germ Free mice and sham inoculated (C) and *P. gingivalis* inoculated (D) Specific Pathogen Free mice. The jaws are stained with methylene blue dye to demarcate the anatomical landmarks used for measuring bone loss (ABC and CEJ). White arrows indicate positions of bone loss measurement.

4.4.1.2. Effect of *P. gingivalis* challenge on the commensal microbiota

Following the 3rd inoculation of SPF mice with *P. gingivalis* there was a highly statistically significant ($p \leq 0.0014$) elevation in the total anaerobic microbial counts of cultivable micro-organisms (Figure 4.16). This elevation was maintained for the experimental period and did not significantly vary following the initial increase ($p \geq 0.05$). There was no significant elevation in anaerobic microbial counts in the sham inoculated control group.

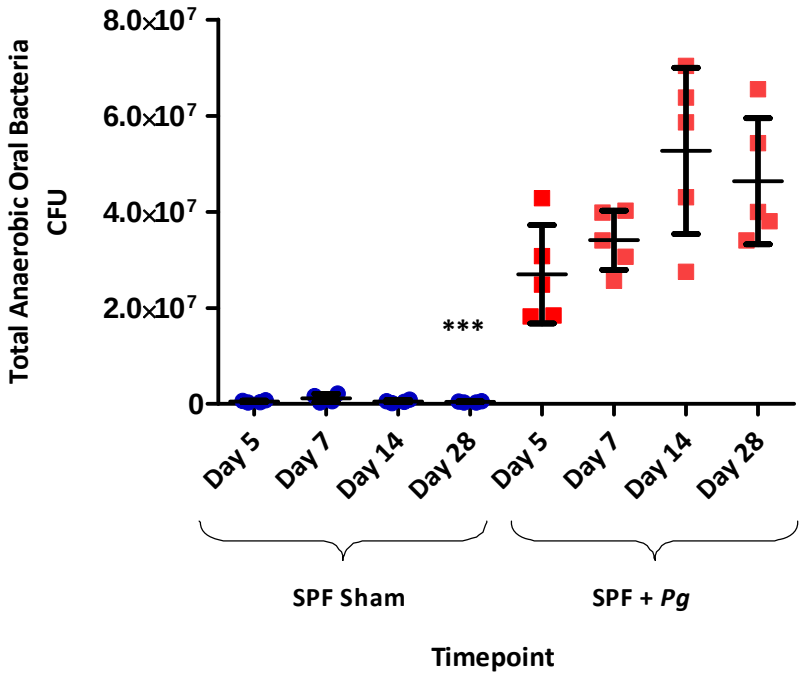

Figure 4.16 Total anaerobic oral bacteria at various time points following inoculation with *Porphyromonas gingivalis* W50 or sham inoculation with carrier vehicle only the mice were assessed for total levels of micro-organisms that were cultivable. The micro-organisms that were cultivable by anaerobic methods are expressed as colony forming units (CFU) data for each individual mouse with horizontal lines denoting the mean values for each group. *** denotes significance at $p<0.0001$. Horizontal lines represent mean values +/- SD.

Accompanying this quantitative increase in the cultivable commensal microbiota from the *P. gingivalis* challenged SPF mice was a change in the components of this microbiota; a qualitative shift (Figure 4.17). The micro-organisms cultivable under aerobic conditions showed a loss of diversity. *Escherichia/Shigella sp* and *Lactobacillus sp* were lost with a concomitant increase in *Enterococcus sp*. Anaerobic cultivation showed a loss of *propionibacterium sp* and *Escherichia/Shigella sp* with the introduction of *Enterococcus sp* following *P. gingivalis* inoculation.

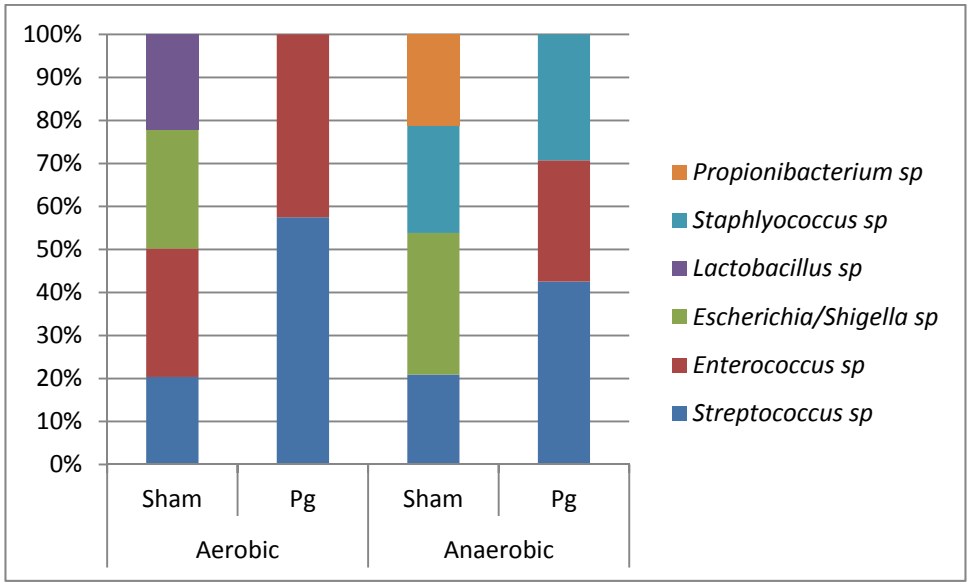

Figure 4.17 Alterations to the cultivable components of the commensal microbiota of SPF mice by aerobic and anaerobic culture methods. These data show the relative composition of the commensal microbiota following sham inoculation with carrier vehicle only or inoculation with *P. gingivalis W50.* Each organism is expressed as log CFU and represented and a percentage of the overall cultivable microbiota. Analysis by 16S partial ribosomal DNA sequencing.

4.4.1.3. Conclusions from *P. gingivalis* challenge of SPF mice from experiment 1

From these results it would seem that a normal intact commensal microbiota is required for periodontal bone loss in this mouse model. Challenging these animals with a known periodontal pathogen, *Porphyromonas gingivalis*, led to further periodontal bone loss but only in those animals with an existing commensal microbiota. Moreover, the inoculation of mice that have a normal commensal microbiota with *P. gingivalis* led to quantitative and qualitative shifts in this commensal microbiota; dysbiosis. This dysbiosis may be the driver of the periodontal bone loss observed.

4.4.2. Results from *P. gingivalis* challenge of SPF mice – experiment 2

If this dysbiosis of the commensal microbiota is the driver of periodontal bone loss these findings were limited by dealing only with the cultivable components. Studies have shown that in the human only around 32% of the oral microbiome is cultivable[112]. Estimates of the cultivable components of the oral microbiome of the mouse are around 50%[416]. The same type of experiment was repeated with a view to looking more closely at the changes in the commensal microbiota focussing on the use of non-culture techniques.

In short, the same protocol was followed for the SPF groups as experiment 1. This time, following microbiological sampling with oral swabs, DNA was extracted. This allowed more detailed analysis of the microbiota using cloning and sequencing techniques.

4.4.2.1. Effect of *P. gingivalis* challenge on alveolar bone Loss

Mice that were inoculated with *P. gingivalis* had highly statistically significant ($p<0.0001$) periodontal bone loss when compared to sham inoculated controls (Figure 4.18). The magnitude of the difference in bone levels was similar to that found previously. (Intra-operator reliability see Appendix 7.3)

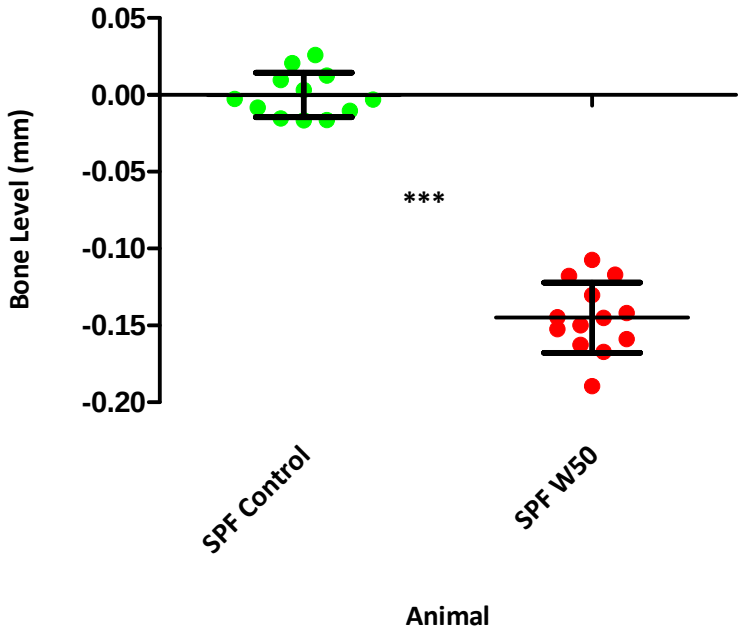

Figure 4.18 Periodontal bone loss expressed as means for each animal with horizontal lines representing the mean value +/- Standard Deviation for each test group. The baseline bone level is that of a wild type SPF mouse i.e. sham inoculated with all bone loss measured relative to this. Bone loss is expressed as negative change in bone relative to this. *** denotes significance at $p<0.0001$. (Intra-operator reliability see Appendix 7.3)

4.4.2.2. Effect of *P. gingivalis* challenge on the commensal Microbiota

Following the 3rd inoculation of SPF mice with *P. gingivalis* there was a highly statistically significant elevation in the total anaerobic microbial counts ($p<0.0001$) and aerobic microbial counts ($p<0.0001$) of cultivable micro-organisms (Figure 4.19 A&B).

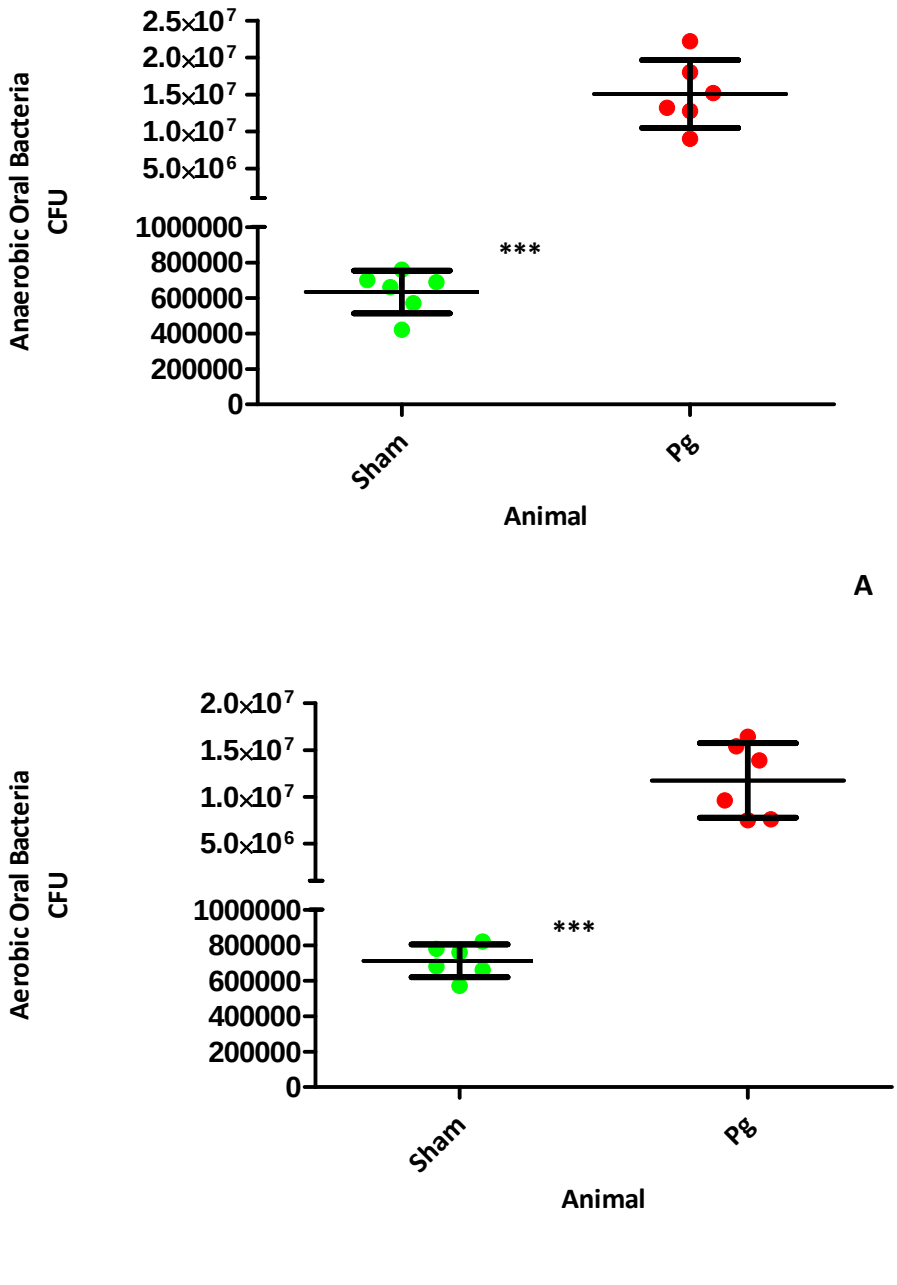

Figure 4.19 Inoculation of C3H SPF mice with *P gingivalis*. Microbial counts of cultivable anaerobic micro-organisms (A) and aerobic micro-organisms (B) cultured from oral swabs taken at day 7 after sham inoculation with carrier vehicle only or inoculation with *P. gingivalis* W50. *** denotes significance at p<0.0001. Horizontal lines represent mean values +/- SD.

From oral swabs taken at day 14 following inoculation with *P. gingivalis*, DNA was extracted and purified. To ensure sufficient quantity of DNA, experimental mice were housed in cages consisting of 4-6 animals. Oral swabs were taken from each mouse and placed into TE. The resultant supernatant was pooled and the DNA extracted to give a representative sample for each cage. Cloning was performed using TOPO-TA cloning and the resultant transformants underwent sequence analysis following conventional Sanger sequencing. Analysis consisted of BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal Database Project website. Sequences were then aligned using ClustalW and trees constructed from distance matrices using the Jukes-Cantor correction by the neighbour-joining method in the MEGAN programme (Appendix 5). Identification was performed at the genus level (Figure 4.20).

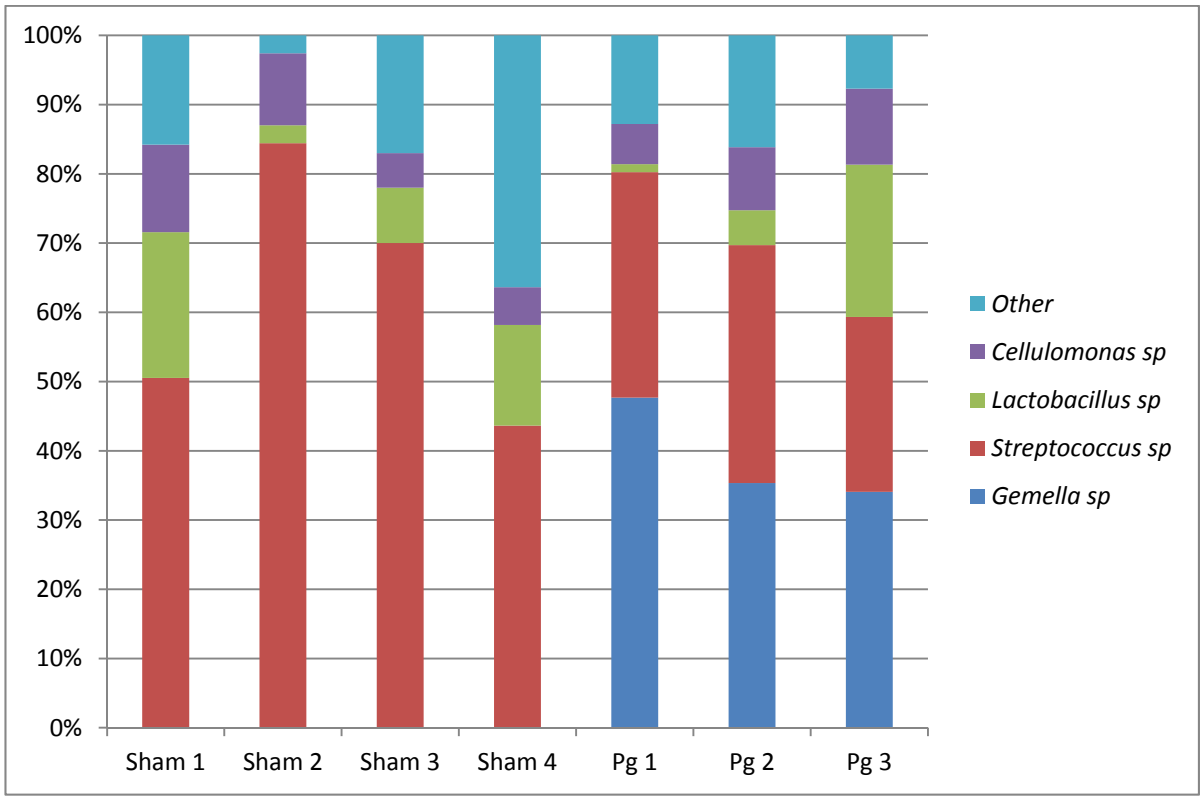

Figure 4.20 Sequence analysis from sham inoculated mice (Sham 1-4) and *P. gingivalis* inoculated mice (Pg 1-3). The 4 major organisms are shown with the remaining organisms represented as 'other'. The sequences are expressed as a proportion (percentage) of the overall number of sequences obtained. All mice were pre-treated with co-trimoxazole.

The only statistically significant compositional alteration to the microbiota was the re-emergence of *Gemella sp*. Despite the graphical appearance (Figure 4.20) that there is a significant change in the proportions of *Streptococcus sp* following *P. gingivalis* inoculation this was not statistically significant (p=0.1214). The remaining major components (*Lactobacillus sp* and *Cellulomonas sp*) and the 'other' components did not differ significantly between sham inoculation and *P. gingivalis* inoculation (p=0.9402, p=0.7210 and p=0.6929 respectively). It is worth remembering that in the oral gavage model of experimental periodontitis all mice are pre-treated with antibiotics (co-trimoxazole) and this, as shown in section 4.3, has significant effects on the composition of the oral commensal microbiota both in terms of microbial counts and composition. These data (Figure 4.20 and 4.21) represent the composition of the oral commensal microbiota 14 days following the 3rd inoculation with *P. gingivalis*. From the data in section 4.1, 14 days is the period of time that transmission of the complete oral commensal microbiota into a germ free animal occurs and so this was considered a reasonable time period for the oral commensal microbiota to stabilise following *P. gingivalis* challenge.

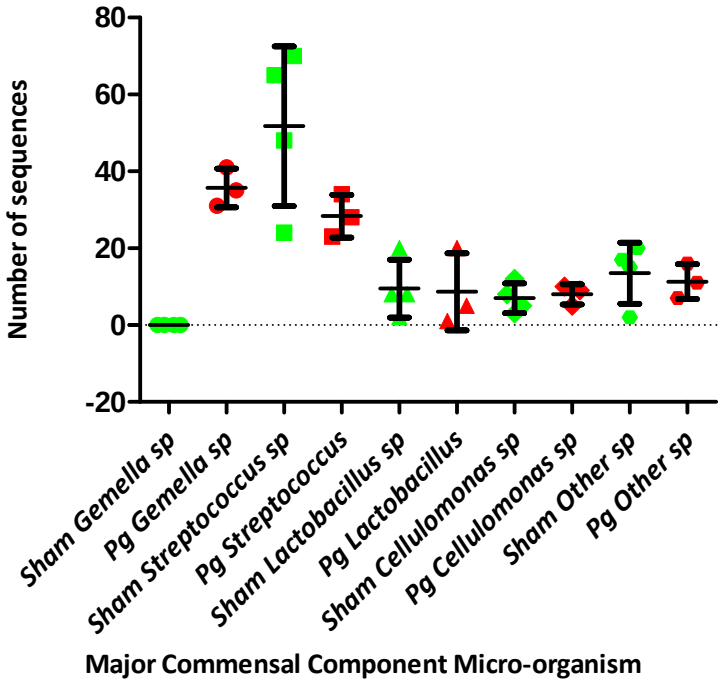

Figure 4.21 The number of sequences of the major commensal microbiota component micro-organisms. Each micro-organism is represented as the number of times that sequence appeared in the sequence analysis. The horizontal lines represent the mean number of sequences for each micro-organism in the sham inoculated mice (green data points) and *P. gingivalis* inoculated mice (red data points).

More detailed analysis of the major commensal component micro-organisms taking a 'best hit' approach on the BLAST database showed that within each genus a number of different strains were present . The *Gemella sp* was uniquely *Gemella palacticanis*-like strain, the *Cellulomonas* a *Cellulomonas hominis*-like strain and the *Lactobacillus sp* was uniquely a *Lactobacillus animalis*-like strain. However, the *Streptococcus sp* showed greater species diversity (Figure 4.22). There were no statistically significant differences between sham and *P. gingivalis* inoculated groups with respect to all species of *Streptococcus sp* ($p>0.05$).

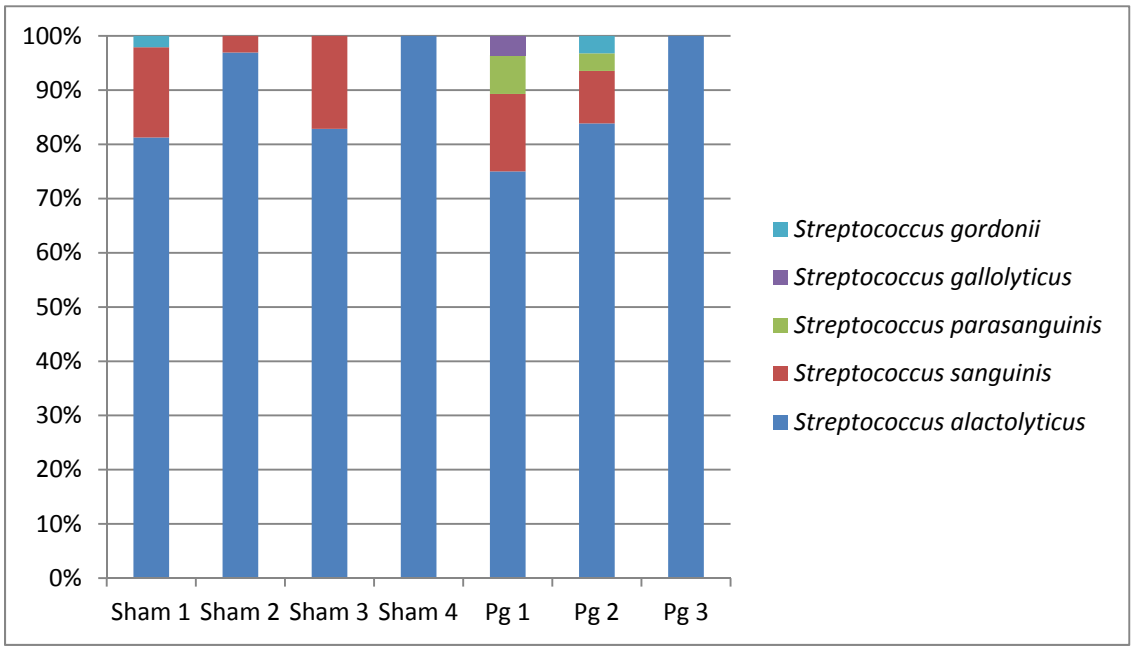

Figure 4.22 Sequence analysis from Sham inoculated and *Pg* inoculated mice showing the species level resolution of *Streptococcus sp*. Each species is expressed as a percentage of the overall sequences obtained for *Streptococcus sp* (22-70 clones per sample).

Components of the commensal microbiota that did not fall into these major genera of micro-organisms were highly varied and diverse (Figure 4.23). Each was only present in very low numbers, typically 1 or 2 cloned sequences per sample. Interestingly, this methodology identified a single clone of a *Porphyromonadaceae* in one of the unchallenged SPF mice (coral band at the top of the left hand bar). Further analysis showed that this was likely to be *Barnesiella sp* with the 'best hit' analysis indicating the greatest homology with *Barnesiella viscericola* (86%) or *Barnesiella intestihominis* (85%) but these identifications were below the assigned threshold of 96% so identification was only possible at the genus level. None of the 'best hits' identifications were *Porphyromonas sp*, so it was unlikely that the source of this micro-organism was from the inoculae.

Figure 4.23 Sequence analysis of the components of the commensal microbiota designated as 'other'. The sham inoculated and *P. gingivalis* inoculated animals sequence data is pooled to give a representative sample for each group. Each sequence is represented as a mean number of clones for each group.

Bacterial genus	Mean Number of Micro-organisms	
	Sham Inoculated	*Pg* Inoculated
Microbacterium sp	2	0.67
Sanguibacter sp	0.75	1
Propionibacterium sp	0	0.33
Flavobacterium sp	0	0.33
Staphylococcus sp	0.5	1
Pseudomonas sp	3	1.33
Acinetobacter sp	1.25	0.33
Clostridium sp	1.5	0
Exiguobacteria sp	0.5	0
Comamonadaceae sp	0.25	0
Agrobacteria sp	0.25	0.33
Massilia sp	0.5	0
Enterobacteria sp	0.5	0
Psychrobacter sp	0.25	0.33
Helicobacter sp	0.25	0
Erysipelotrichaceae sp	0.5	0.33
Bacteroides sp	2.5	0
Barnesiella sp	0.5	0
Roseburia sp	0.75	0
Oscillibacter sp	0.25	0
Hydrogenoanaerobacterium sp	0.25	0
Lachnospiraceae sp	0.75	0
Ruminococcaceae sp	0.5	0
Porphyromonadaceae sp	0.25	0
Paracoccus sp	0	0.33
Duganella sp	0	0.33
Stentrophomonas sp	0	0.33
Oxalobacteriaceae sp	0	0.33

4.4.3. Detection of *P. gingivalis*

Porphyromonas gingivalis is a black pigmenting Gram-negative rod. Following oral sampling anaerobic cultures were kept for a period of 2 weeks to assess the formation of black pigmenting colonies. At no point were colonies of this description seen.

A non-quantitative technique was used for assessing the presence of *P. gingivalis*. Oral swabs were 'smeared' across a microscope slide and detection was possible using monoclonal antibody 1B5 (MAb 1B5) followed by a fluorescein isothiocyanate (FITC) labelled anti-mouse secondary antibody[421] (section 3.6). Counter staining with 4', 6-diamidino-2-phenylindole (DAPI) allowed comparison to all bacterial cells present in the oral swab (Figure 4.24 A, B&C).

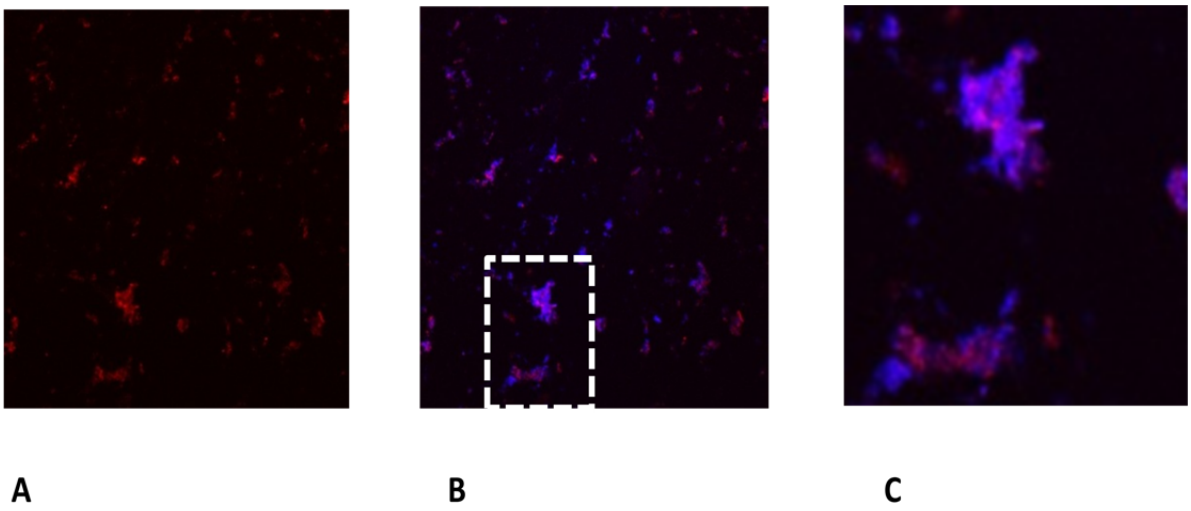

Figure 4.24 Detection of *P. gingivalis* in oral swabs of challenged SPF mice by immuno-fluorescence microscopy. *P. gingivalis* was detected using MAb 1B5 followed by a Texas Red-labelled anti-mouse secondary antibody. Immunolabelled *P. gingivalis* from an oral swab from SPF mouse 20 days after inoculation (A). Same field counterstained with DAPI (B) showing all bacterial cells present. Expanded view (C) of boxed region showing aggregates of bacteria containing *P. gingivalis.* Slide courtesy of Dr Asil Alsam.

In addition, PCR was performed on the samples using a 16S rRNA gene species-specific PCR oligonucleotide primers. Genomic DNA extracted from parental *P. gingivalis* served as positive control with no template DNA as negative control. The resultant product was developed on a gel of 1% agarose in TBE with ethidium bromide (Figure 4.25 A&B).

P. gingivalis was detected in the pooled DNA following PCR amplification of the 16S rRNA gene from all the cages that contained mice that were inoculated by oral gavage (lanes 9-16). In addition, *P. gingivalis* was not detected in the pooled DNA from the cages containing mice that were 'sham' inoculated with carrier vehicle only (lanes 1-8). Lane 17 contained DNA from *P. gingivalis* altered microbiota experiment (section 4.6.). The quantity of DNA present in each sample was highly variable.

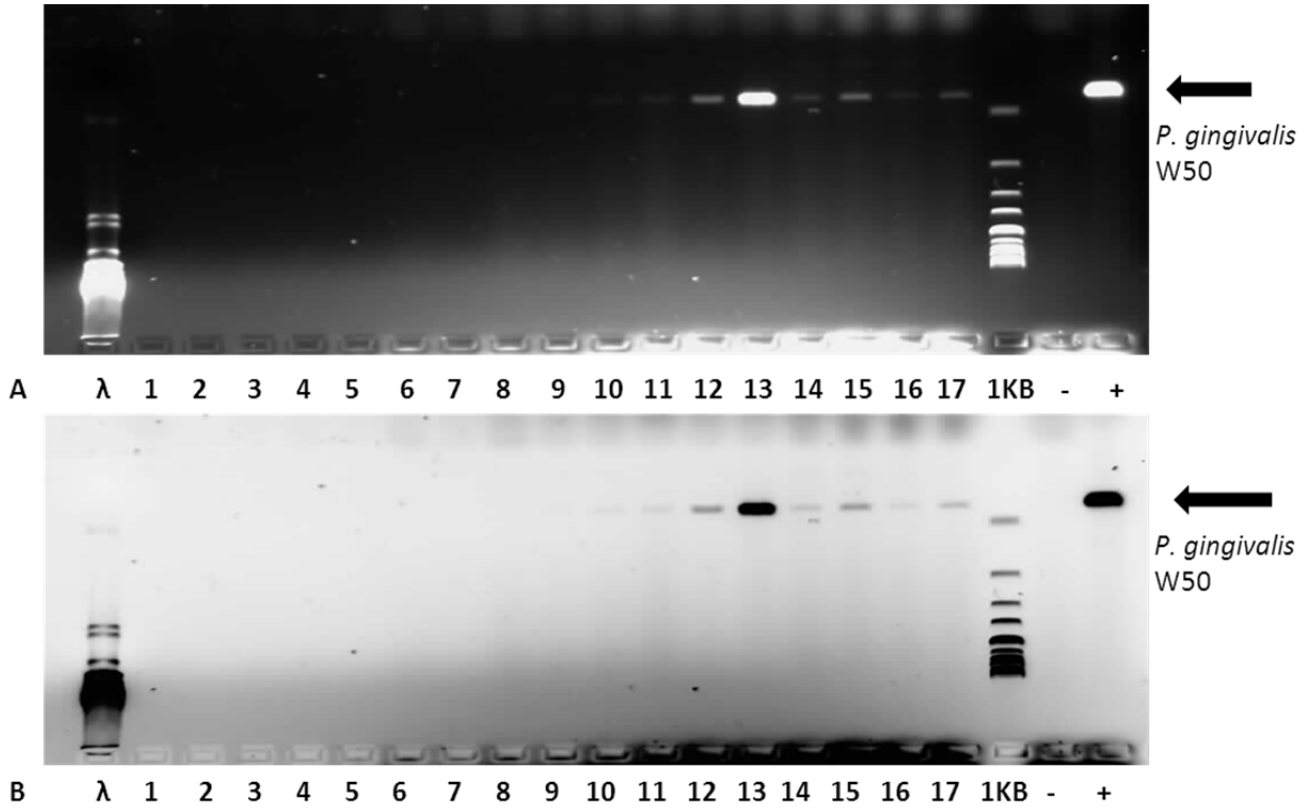

Figure 4.25 Agarose gel of amplicons of *P. gingivalis* specific PCR under UV transillumination (A) and the negative image (B). Lanes 1-8 are samples from cages of mice not inoculated with *P. gingivalis*, lanes 9-16 are samples from cages of mice inoculated with *P. gingivalis*. Lane 17 is from a cage of mice into which a *P. gingivalis* altered microbiota has transferred (see section 4.6. *P. gingivalis* altered commensal microbiota transmission experiment). Ladders are 1Kb and λ respectively and the lanes to the far right are negative and positive controls respectively.

4.4.4. Tartrate resistant alkaline phosphatase (TRAP) staining for osteoclasts

The macroscopic appearance of the alveolar bone suggests a bone resorptive process rather than a failure to form the bone. Such features include irregular alveolar bone crests indicative of resorption and vertical patterns of bone loss localised to individual roots of individual teeth. This is very much akin to the pattern of inflammatory alveolar bone loss in human periodontal disease.

In an attempt to further confirm this, 10μm sections were taken of decalcified periodontal tissues to examine them for histological features of bone resorption along with TRAP staining to identify the presence of activated osteoclasts and active bone resorption. The sections were taken from mice 6 weeks after challenge with *P. gingivalis* at sacrifice.

Activated osteoclasts were rarely seen which is not surprising given that the samples were prepared at 6 weeks after inoculation and bone resorption may be an early phenomenon (Figures 4.26 & 4.27). There were microscopic signs of bone resorption such as scalloping and irregularity of the alveolar bone crest and coronal most parts of the supporting bone. In addition, there was evidence of reversal lines deeper within the alveolar bone structure which are indicative of historical bone resorption (Figures 4.28 & 4.29). No significant differences were found between *P. gingivalis* challenged and unchallenged mice in terms of osteoclast numbers or microscopic features of bone resorption.

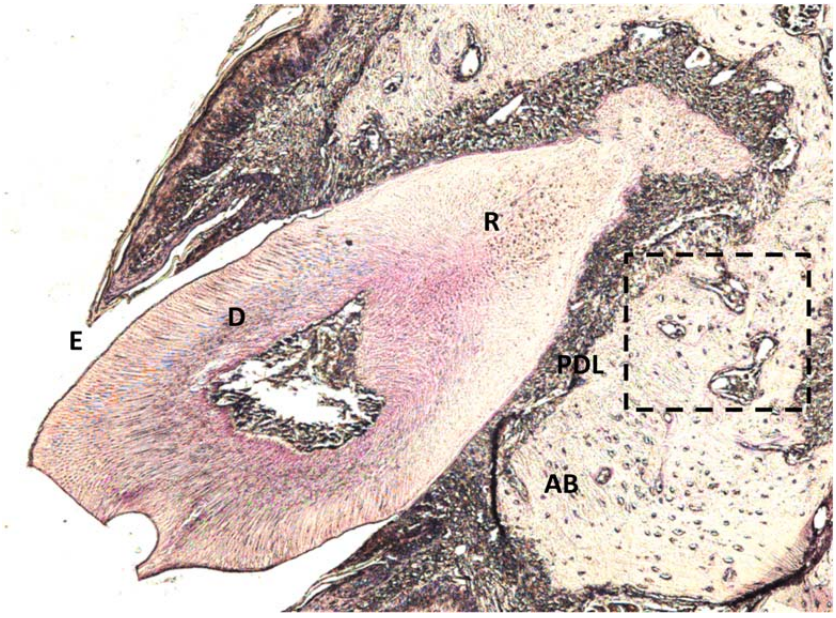

Figure 4.26 Transverse 10µm section through the first molar tooth of a *P.gingivalis* challenged C3H SPF mouse showing tooth (enamel space E and dentine D), tooth root (R), periodontal ligament (PDL) and alveolar bone (AB). The section is TRAP stained then counter stained with haemotoxylin. The area highlighted by the box is magnified in Figure 4.26 to show osteoclasts.

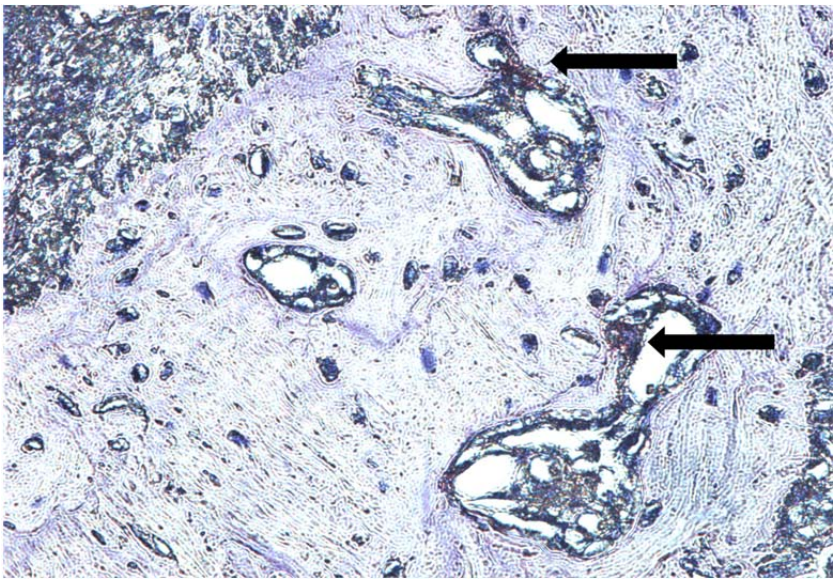

Figure 4.27 Magnification of the area highlighted in of the same *P. gingivalis* challenged SPF C3H mouse in Figure 4.25 showing the presence of osteoclasts, staining red with TRAP (highlighted by black arrows), in deeper bone structure.

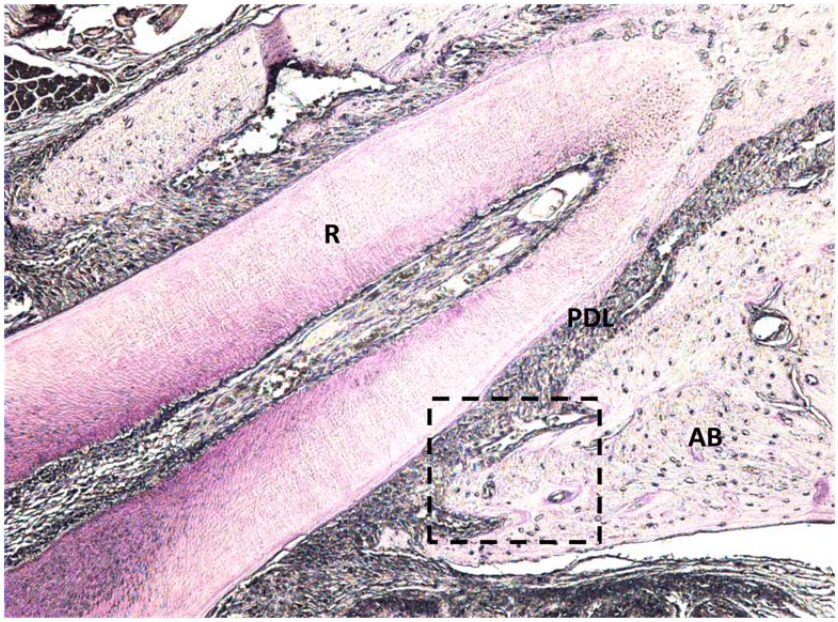

Figure 4.28 Transverse 10µm section through the first molar tooth of a *P.gingivalis* challenged C3H SPF mouse which was TRAP stained then counter stained with haemotoxylin. The section shows the tooth root (R), periodontal ligament (PDL) and alveolar bone (AB). The area of alveolar bone highlighted in the box is magnified in Figure 4.28 to show microscopic features of bone resorption.

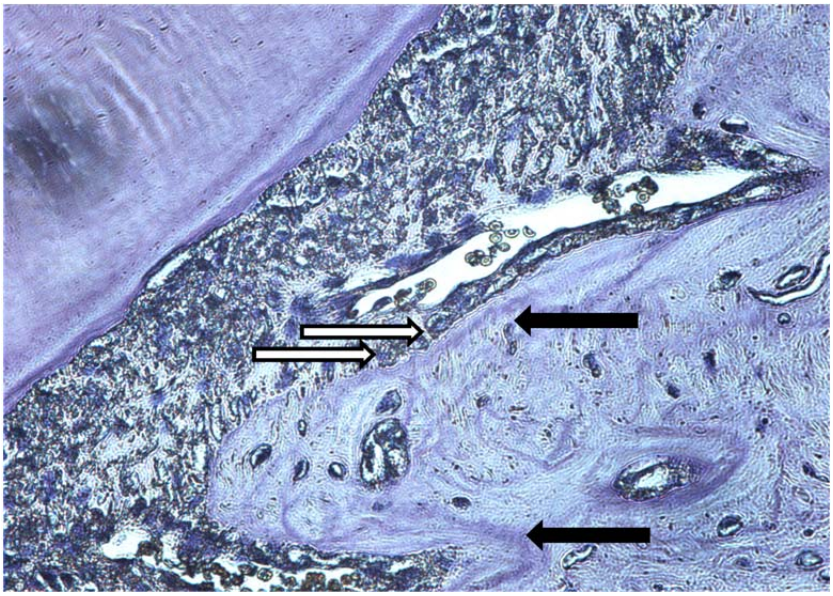

Figure 4.29 Magnification of the area highlighted in of the same *P. gingivalis* challenged SPF C3H mouse in Figure 4.27 showing microscopic evidence of bone resorption. Surface scalloping is highlighted by the white arrows and reversal lines highlighted by the black arrows.

4.5. Commensal microbiota transmission experiments

An intact commensal microbiota was necessary for accelerated periodontal bone loss to occur irrespective of the presence of *P. gingivalis*. In the absence of this microbiota, in a Germ Free (GF) animal, no periodontal bone loss occurs. This led to the question as to whether germ free animals would develop alveolar bone loss when an oral commensal microbiota was introduced. This was investigated in a series of co-caging experiments.

In short, female SPF mice with a known microbiota were housed in the same cages as GF mice in a ratio of 1:2. The transmission of the commensal microbiota was then monitored over time by culture dependent methodology. Bone loss was then assessed by morphological means after the usual experimental period of 6 weeks but also after an extended period of co-caging of 16 weeks.

We know from the results of the initial establishment of the 2 colonies of C3H/Orl mice that the germ free animals receive this commensal microbiota completely and within 14 days (section 4.1.1.). This was assessed by culture-dependant and non-culture-dependant techniques.

4.5.1. Transmission of commensal microbiota using culture techniques

4.5.1.1. Total microbial counts of the oral commensal microbiota

Oral swabs were taken from the SPF C3H/Orl at baseline, day 14 and at the end of the co-caging period after 16 weeks. In addition, swabs were taken from the GF C3H/Orl mice on delivery (Day 0) and at day 14 and 16 weeks. These swabs were stored in John's transport media and processed for culture by vortexing to release the bacteria from the swab into the transport media. Serial dilutions were made of this supernatant and these were plated onto blood agar and incubated aerobically and anaerobically for 48 hours. Colony counts were then performed.

Using these culture techniques it was confirmed that no micro-organisms were detectable in the germ free mice on delivery when samples were incubated under aerobic and anaerobic conditions (Figure 4.30 and 4.31). The baseline levels of micro-organisms in the SPF mice were not significantly different from those at the time points day 14 and week 16 under both sets of incubation conditions. At day 14 the aerobic and anaerobic counts were not statistically significantly different from the SPF mice at this time point (p=0.6978 and p=0.9367 respectively). In addition, these levels were then maintained with no statistically significant differences for the extended period of co-caging to week 16.

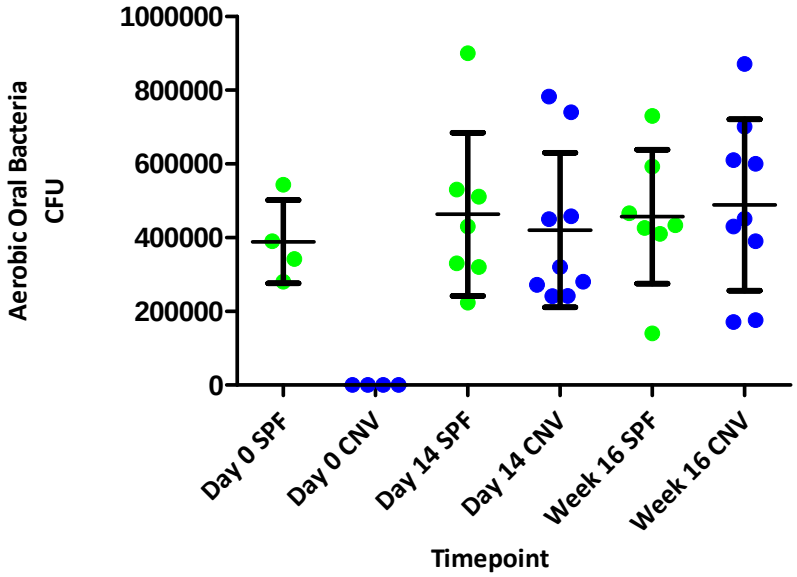

Figure 4.30 Aerobic bacterial counts in colony forming units (CFU) for C3H/Orl SPF mice (green dots) and C3H/Orl conventionalized mice (Blue dots, CNV – previously germ free mice that were co-caged with SPF mice). There were no statistically significant differences between the SPF mice at any time points or with the CNV mice after the initial 14 days of co-caging. Horizontal lines represent mean values +/- SD.

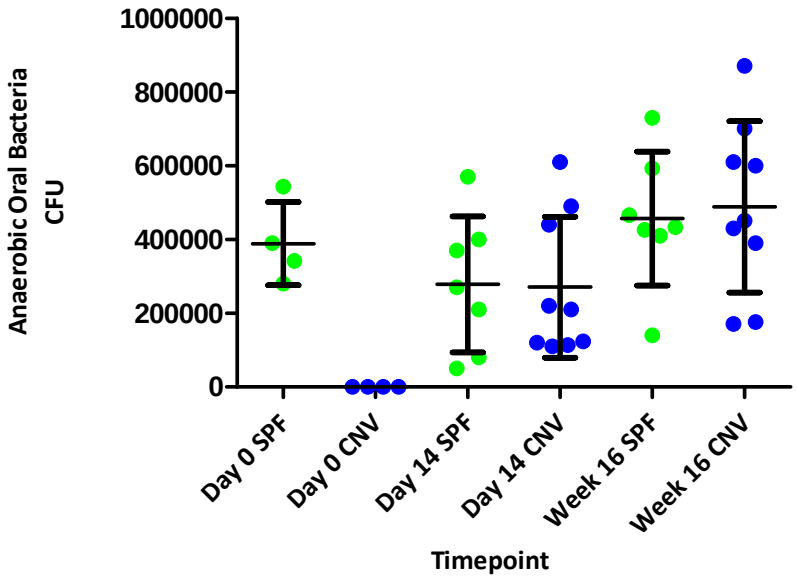

Figure 4.31 Anaerobic bacterial counts in colony forming units (CFU) for C3H/Orl SPF mice (green dots) and C3H/Orl conventionalized mice (Blue dots, CNV – previously germ free mice that were co-caged with SPF mice). There were no statistically significant differences between the SPF mice at any time points or with the CNV mice after the initial 14 days of co-caging. Horizontal lines represent mean values +/- SD.

4.5.1.2. Composition of the commensal oral microbiota

From the agar plates produced by serial dilution of the supernatant in which the oral swabs were placed colonies were distinguished based on morphological differences. These individual colonies were then isolated and subcultured onto fresh blood agar. Identification of these individual colonies was performed using 16S rRNA sequencing and interrogation of the sequences against the NCBI BLAST database.

There were 3 major cultivable micro-organisms under both aerobic and anaerobic conditions. Aerobically the major components of the oral commensal microbiota were *Streptococcus sp*, *Lactobacillus sp* and *Escherichia/Shigella sp* (Figure 4.32). The major anaerobic cultivable micro-organisms were *Streptococcus sp*, *Lactobacillus sp* and *Staphylococcus sp* (Figure 4.33). Consistent with the previous findings regarding transmission of the oral commensal microbiota from an SPF mouse into a GF mouse the major cultivable micro-organisms were transmitted across into the germ free mice by day 14 and in similar proportions. This microbiota is then stable in the conventionalised mice throughout the 16 weeks of co-caging.

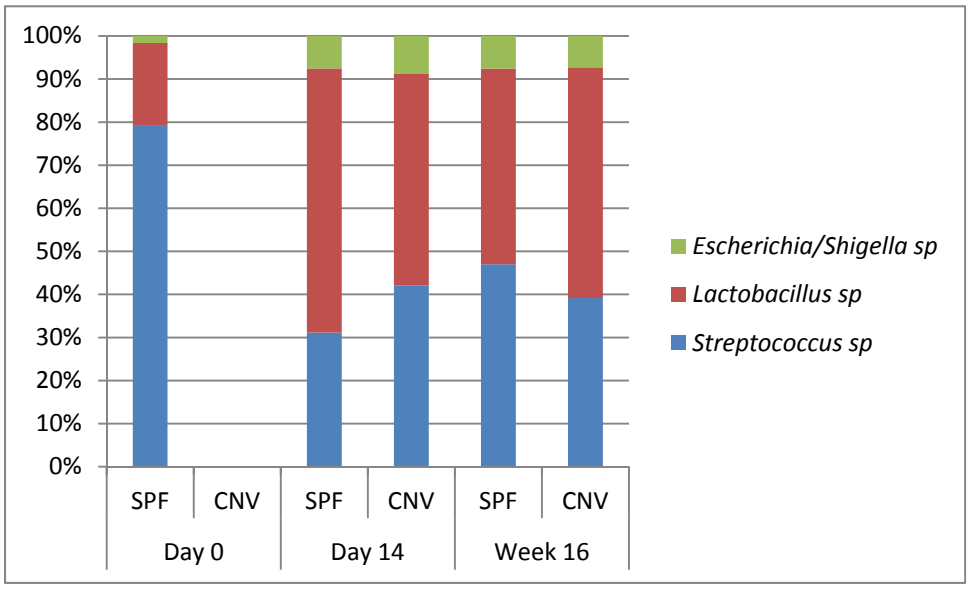

Figure 4.32 Composition of the oral commensal microbiota of SPF mice and conventionalised mice (CNV – previously germ free mice that were co-caged with the SPF mice) cultivable under aerobic conditions. Each species of micro-organism was estimated as colony forming units (CFUs) and expressed as their proportion (percentage) of the total number of CFU detected.

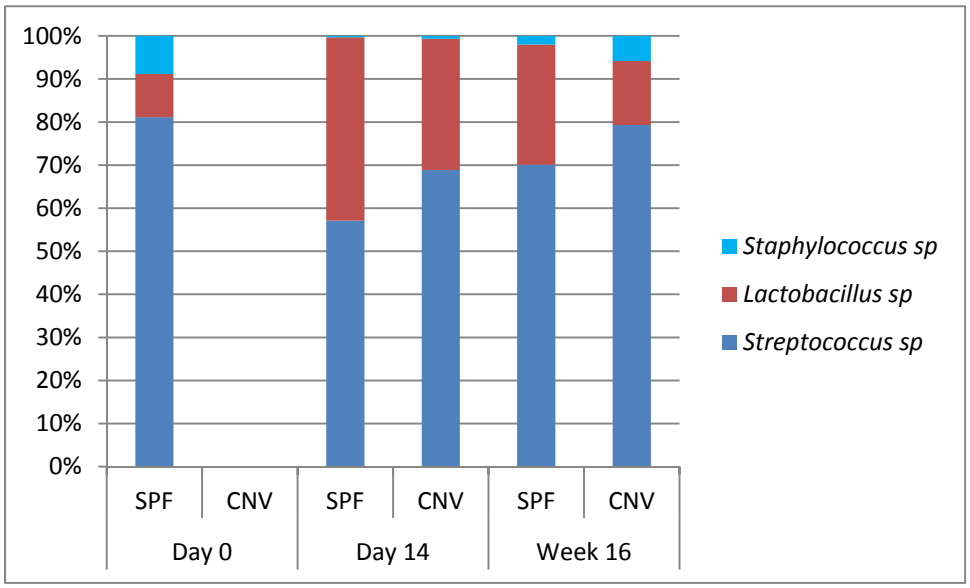

Figure 4.33 Composition of the oral commensal microbiota of SPF mice and conventionalised mice (CNV – previously germ free mice that were co-caged with the SPF mice) cultivable under anaerobic conditions. Each species of micro-organism was estimated as colony forming units (CFUs) and expressed as their proportion (percentage) of the total number of CFU detected.

4.5.2. Composition of the oral commensal microbiota using non-culture techniques

Further investigation of the composition of the oral commensal microbiota was carried out using conventional 16S rRNA sequencing. From oral swabs DNA was extracted and the 16S rRNA gene amplified by PCR (section 3.5.3.). Following cloning into *E coli* random transformants were selected for re-amplification of the flanking M13 regions and sequencing. Analysis consisted of BLAST interrogation of the GenBank nucleotide database and the Sequence Match algorithm on the Ribosomal Database Project website. Sequences were then aligned using ClustalW and trees constructed from distance matrices using the Jukes-Cantor correction by the neighbour-joining method in the MEGAN software (Appendix 5).

The results were presented in section 4.1.3. with complete transmission of the oral commensal microbiota from SPF mice into CNV mice (shown in figures 4.4, 4.5 and 4.6). In summary, 3 major bacterial species that were consistently detected were *Lactobacillus sp*, *Streptococcus sp* and *Gemella sp*. More detailed analysis of the major microbial species present was performed based on a 'best hit' approach using the BLAST database. This gives identification for each genera based on the greatest degree of homology of the sequence with the database over the largest part of the sequence. This approach showed that the *Gemella sp* was exclusively a *Gemella palacticanis*-like micro-organism and the *Lactobacillus sp* was largely a *Lactobacillus animalis*-like micro-organism. This analysis showed that there were 2 different strains of *Streptococcus sp* that made up the majority of this species; a *Streptococcus alactolyticus*-like and a *Streptococcus sanguinis*-like species. In addition, there were *Streptococcus intermedius*, *parasanguinis*, *equi*, *salivarius* and *peroris* – like organisms present in very low numbers.

4.5.3. Alveolar Bone Loss

Specific Pathogen Free (SPF) C3H mice are able to fully transmit the commensal microbiota to Germ Free (GF) C3H mice. This occurs completely within 14 days. Complete transmission of the commensal microbiota led to periodontal bone loss but at a delayed rate (Figure 4.34). By the usual experimental period of 6 weeks, despite complete 'infection' with a normal SPF commensal microbiota, GF mice have bone levels similar to GF controls. It is only after a prolonged period of cocaging, 16 weeks, that they develop bone loss to a degree similar to age matched control SPF mice. (Intra-operator reliability see Appendix 7.4).

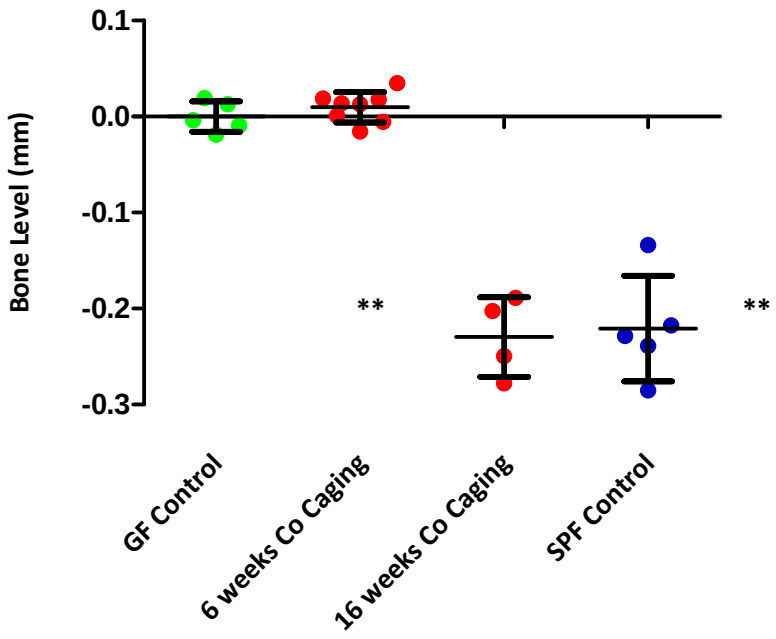

Figure 4.34 Bone levels for GF C3H mice cocaged with SPF C3H mice at 6 and 16 weeks after cocaging. Negative values indicate bone loss relative to mice maintained in germ free conditions (GF Control) for the entire experimental period. Data are mean bone level per animal with horizontal lines representing the mean values for each group +/- SD. ** represents significance at $p<0.001$ level when compared to GF control. (Intra-operator reliability see Appendix 7.4)

4.6. Transmission of a *P. gingivalis* altered commensal microbiota from SPF into GF mice

The normal commensal microbiota is necessary for periodontal bone loss to occur and this microbiota is transmissible from an SPF animal into a GF animal of the same strain. There is also evidence that challenging this normal commensal microbiota with a known periodontal pathogen, *Porphyromonas gingivalis*, causes a quantitative and qualitative shift in this microbiota. The question then arises as to whether this commensal microbiota, 'altered' by challenge with *P. gingivalis*, would be transmissible into a GF animal and whether this 'altered' microbiota would then result in periodontal bone loss to the same extent.

This hypothesis was tested in another co-caging experiment. In short, female SPF mice were challenged with *P. gingivalis* in the standard oral gavage protocol; 3 inoculations at 48 hours apart. Oral swabs from these animals were then taken to provide both cultural and sequencing determination that the microbiota had experienced a quantitative and qualitative shift; become 'altered'. Once this was confirmed GF mice were co-caged with these SPF animals with *P. gingivalis* altered microbiota and monitoring of the transmission of this 'altered' microbiota was performed. Bone loss analysis was performed following sacrifice.

4.6.1. Quantitative alteration of commensal microbiota by *P. gingivalis* challenge

The increase in microbial load of the commensal microbiota was confirmed by culturing oral swabs under aerobic and anaerobic conditions. The resultant colonies were counted (Figure 4.35 and 4.36). Under both sets of conditions there was a highly significant increase in total aerobic ($p<0.001$) and anaerobic counts ($p<0.001$) 7 days following challenge with *P. gingivalis* (Baseline SPF compared to Day 0 SPF + *Pg*). At this time point (Day 0) co-caging began, the germ free animals (called conventionalized CNV) having no commensal microbiota when detected by cultural means under aerobic and anaerobic conditions (Day 0 CNV). Following 14 days of co-caging the CNV animals had microbial counts that were not statistically different ($p>0.1$) from the SPF mice

following challenged with *P. gingivalis* at the initial time point (Day 0) or the current time point (Day 14).

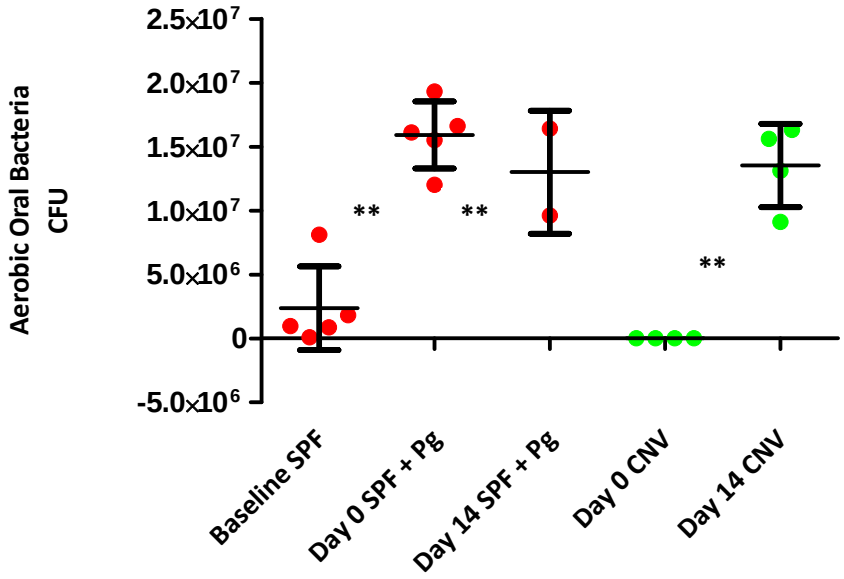

Figure 4.35 Total aerobic microbial counts taken at start of experiment (Baseline SPF), following inoculation of SPF mice with *P. gingivalis* and start of co-caging with CNV mice (Day 0) and following 14 days of co-caging (Day 14). These data are expressed as CFU of total cultivable aerobic micro-organisms. The counts are expressed as a total for each animal with horizontal lines representing means +/- SD of CFU for SPF mice (red points) and conventionalized (CNV) mice (green points). ** represents statistical significance $p<0.001$ from baseline SPF mice.

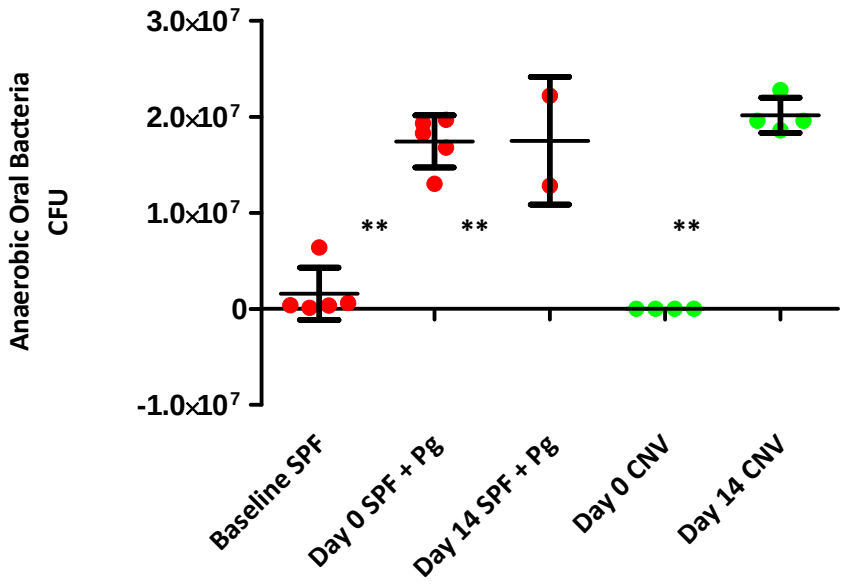

Figure 4.36 Total anaerobic microbial counts taken at start of experiment (Baseline SPF), following inoculation of SPF mice with *P. gingivalis* and start of co-caging with CNV mice (Day 0) and following 14 days of co-caging (Day 14). These data are expressed as CFU of total cultivable anaerobic micro-organisms. The counts are expressed as a total for each animal with horizontal lines representing means +/- SD of CFU for SPF mice (red points) and conventionailsed (CNV) mice (green points). ** represents statistical significance p<0.001 from baseline SPF mice.

4.6.2. Qualitative alteration of commensal microbiota by *P. gingivalis* challenge

Sequence analysis showed that the commensal microbiota underwent a qualitative shift in its composition as a result of challenge with *P. gingivalis* when compared to unchallenged control mice (Figure 4.37). The challenged and unchallenged mice were pre-treated with co-trimoxazole in accordance with the experimental protocol. The effect of this was to eliminate the *Gemella sp* from the oral commensal microbiota with concurrent increases in mainly *Streptococcus sp*. Following challenge with *P. gingivalis* the *Gemella sp* was able to re-establish as a major component of the oral commensal microbiota. It was this 'altered flora' that was transmitted to the previously germ free mice in terms of the major component microorganisms.

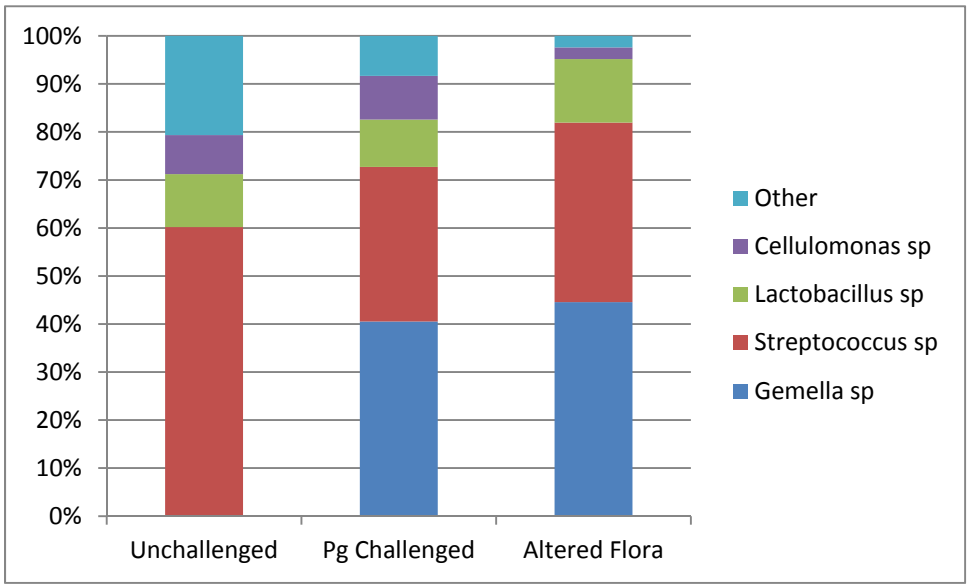

Figure 4.37 Challenge of SPF mice with *P. gingivalis* results in qualitative shifts in the commensal microbiota (compare unchallenged with *Pg* challenged). This 'altered flora' is transmissible into a previously germ free animal (altered flora). These data are based on sequence analysis of a random sample of clones for each experimental group and are expressed as total number of sequences for each group as a percentage of the overall composition of the microbiota.

There was however, a significantly reduced transmission of the component micro-organisms classified as 'other' (Figure 4.38). The co-caging process resulted in the transmission of only 2 of the' other' component micro-organisms into the conventionalised mice; namely *Staphylococcus sp* and *Veillonella sp*. Indeed, the *P.gingivalis* challenged mice (altered flora) had 14 different micro-organisms represented in the 'other' group in 1 to 5 clones per animal whereas the conventionalised mice (previously germ free) only received these two micro-organisms (*Staphylococus sp* and *Veillonella sp*) represented as single clones. This reduced diversity of transmission of the 'other' components may be a real effect in which only the certain constituents of this altered microbiota are transmissible. However, previous transmission experiments have shown that this has not been the case so it is more likely a methodological phenomena e.g. the major components may have been over-represented in the transformants and consequently preferentially selected during the random selection of transformants.

The *Porphyromonadaceae* detected in the non challenged group is the same transformant as discussed previously and is likely to be *Barnesiella sp* and an incidental finding rather than a *Porphyromonadaceae* introduced directly by the *P. gingivalis* inoculation process.

Bacterial genus	Mean Number of Micro-organisms		
	Sham Inoculated	*Pg* Inoculated	Altered Flora
Microbacterium sp	2	0.67	0
Sanguibacter sp	0.75	1	0
Propionibacterium sp	0	0.33	0
Flavobacterium sp	0	0.33	0
Staphylococcus sp	0.5	1	0.25
Burkholderia sp	0	0	0
Pseudomonas sp	3	1.33	0
Acinetobacter sp	1.25	0.33	0
Clostridium sp	1.5	0	0
Exiguobacteria sp	0.5	0	0
Comamonadaceae sp	0.25	0	0
Agrobacteria sp	0.25	0.33	0
Massilia sp	0.5	0	0
Enterobacteria sp	0.5	0	0
Psychrobacter sp	0.25	0.33	0
Helicobacter sp	0.25	0	0
Erysipelotrichaceae sp	0.5	0.33	0
Bacteroides sp	2.5	0	0
Barnesiella sp	0.5	0	0
Roseburia sp	0.75	0	0
Oscillibacter sp	0.25	0	0
Hydrogenoanaerobacterium sp	0.25	0	0
Lachnospiraceae sp	0.75	0	0
Ruminococcaceae sp	0.5	0	0
Porphyromonadaceae sp	0.25	0	0
Paracoccus sp	0	0.33	0
Duganella sp	0	0.33	0
Stentrophomonas sp	0	0.33	0
Oxalobacteriaceae sp	0	0.33	0
Veillonella sp	0	0	0.25

Figure 4.38

Figure 4.38 Sequence analysis of the 'other' minor components of the microbiota of unchallenged and *P. gingivalis* challenged mice along with transmission of this microbiota to previously germ free mice (altered flora). These data are based on pooled DNA from each experimental group (n=4 to n=6) and sequence analysis of a random sample of clones for each experimental group and are expressed as mean number of sequences for each group.

4.6.3. Alveolar bone Loss

Transmisson of the 'altered flora' to previously germ free (conventionalised) animals leads to highly statistically significant (P<0.0001) periodontal bone loss after 16 weeks of co-caging when compared to animals with unaltered microbiota, sham inoculated SPF controls (Figure 4.39). The level of bone loss is similar to age matched SPF animals challenged with *P. gingivalis* (p=0.6107). (Intra-operator reliability see Appendix 7.5).

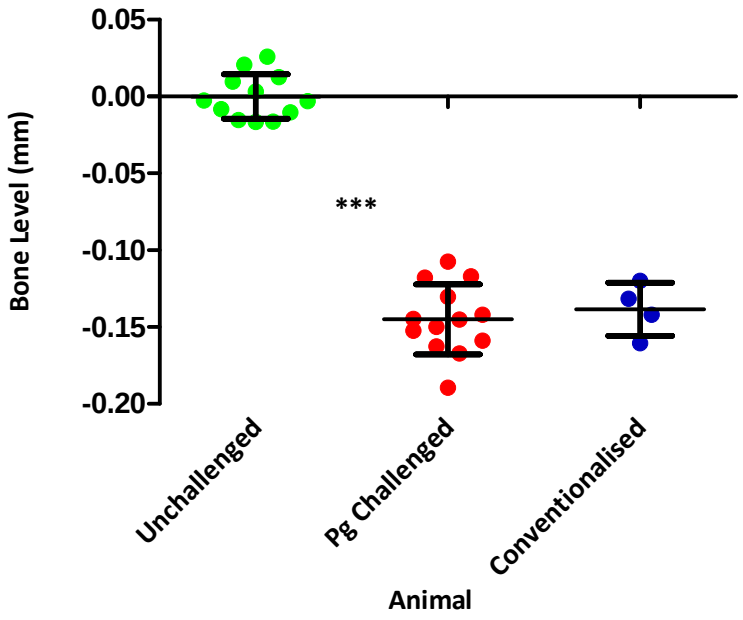

Figure 4.39 SPF mice challenged with *P. gingivalis* show significantly more periodontal bone loss when compared to unchallenged SPF control mice. The dysbiotic microbiota from these *P.gingivalis* challenged mice was transmissible into a previously germ free (conventionalised) animal and led to increased levels of periodontal bone loss when compared to unchallenged SPF controls. The level of bone loss was similar to age matched *P. gingivalis* challenged SPF animals. Bone loss is presented for each animal relative to unchallenged SPF control mice with horizontal lines representing the mean level +/- SD. *** shows significance P<0.0001. (Intra-operator reliability see Appendix 7.5)

4.7. Influence of mouse strain on the commensal microbiota

All experiments to date have been confined to a single mouse strain; namely C3H/Orl. In order to examine the influence of the mouse genotype on the commensal microbiome we carried out a series of co-caging experiments whereby Specific Pathogen Free (SPF) mice in a Balb/cAnNCrl background were co-aged with Germ Free (GF) in a C3H/Orl background. The transmission of the commensal microbiota was monitored by culture and non-culture techniques as previously described and ultimately bone levels were assessed after 16 weeks of co-caging.

4.7.1. Defining the commensal microbiota of the SPF Balb/cAnNCrl mouse

4.7.1.1. Microbial counts of the commensal microbiota

There were no statistically significant differences between the total aerobic (Figure 4.40) and anaerobic (Figure 4.41) microbial counts between SPF C3H/Orl and Balb/cAnNCrl mice.

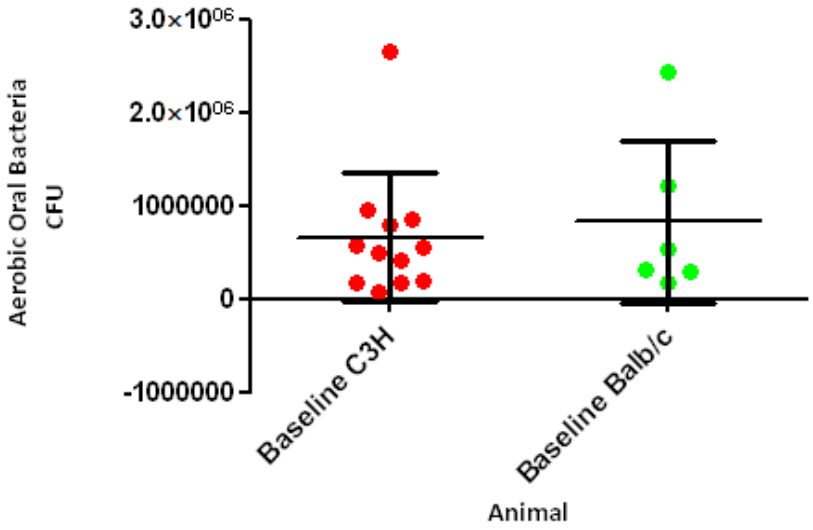

Figure 4.40 Total aerobic microbial counts for an SPF C3H/Orl and Balb/cAnNCrl mouse. Data expressed as total number of colony forming units (CFU) for each animal with horizontal lines representing mean values for each strain of mouse +/- SD.

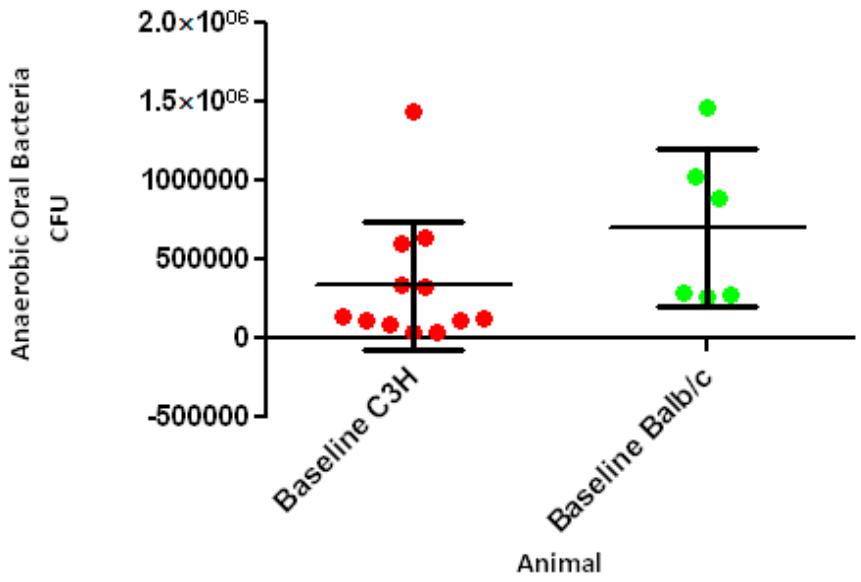

Figure 4.41 Total anaerobic microbial counts for an SPF C3H/Orl and Balb/cAnNCrl mouse. Data expressed as total number of colony forming units (CFU) for each animal with horizontal lines representing mean values for each strain of mouse +/- SD.

4.7.1.2. Composition of the Balb/cAnNCrl commensal microbiota using culture techniques

Oral swabs from both C3H/Orl and Balb/cAnNCrl mice were cultured for 48 hours on blood agar under aerobic and anaerobic conditions. Colonies were distinguished based on morphology and colony counts performed. Individual morphological types were isolated and sub-cultured. The DNA from these isolates was extracted and identification of bacteria was made by amplification of the 16S rRNA gene and comparison to the NCBI BLAST database.

Based on culture techniques and morphological identification there were no significant differences in the components of the commensal microbiota between C3H/Orl and Balb/cAnNCrl mice (Figure 4.42). The major aerobic components of the Balb/c oral commensal microbiota detectable by culture means were *Streptococcus sp*, *Lactobacillus sp* and *Escherichia/Shigella sp* which is comparable with those found in the C3H/Orl mice. In terms of the major anaerobic components of the Balb/c oral commensal microbiota those cultivable were *Streptococcus sp*, *Lactobacillus sp* and *Staphylococcus sp* which was similar to the major cultivable anaerobic micro-organisms in the C3H/Orl mice.

	Aerobic		Anaerobic	
	Balb/c	**C3H**	**Balb/c**	**C3H**
Streptococcus sp	331666.7	166666.7	0	0
Lactobacillus sp	375000	283333.3	0	0
Escherichia/Shigella sp	850	750	0	0
Staphylococcus sp	1000	0	0	0
Streptococcus sp	0	0	513333.3	150000
Lactobacillus sp	0	0	221250	345000
Staphylococcus sp	0	0	0	1000

Figure 4.42 Aerobic and anaerobic isolates of the oral swabs from C3H/Orl and Balb/c mice were identified by 16S rRNA PCR of pooled DNA from cages containing 4-6 mice from each strain. Identification was performed by interrogation of sequences against the NCBI BLAST database. Each colony was isolated based on morphological differences. Data are expressed mean values of CFU/ml for each group of animals.

4.7.1.3. Composition of the Balb/cAnNCrl commensal microbiota using non-culture techniques

DNA was extracted from oral swabs from C3H/Orl and Balb/cAnNCrl mice and pooled for each of the cages, 3 cages of Balb/c mice and 4 cages of C3H mice each with 2-6 mice per cage, and the 16S gene amplified by PCR. The resultant amplicons were cloned into *E coli* then random selections of up to 95 transformants were sequenced from each cage. Identification of these clones was made by interrogation of the BLAST database and trees constructed using MEGAN4 (Appendix 5).

The same 4 major species were present in both C3H and Balb/c mice (Figure 4.43) although their proportions in the commensal microbiota of each mouse strain appeared to be different. These were *Gemella sp*, *Streptococcus sp*, *Lactobacillus sp* and *Cellulomonas sp* with a significant group of micro-organisms in the 'other' group. More detailed analysis of the Balb/c major components of the oral microbiota, based on a 'best hit' approach using the NCBI BLAST database, showed that these micro-organisms were exclusively *Gemella palacticani*-like or *Cellulomonas persica*-like. There was greater diversity in the *Lactobacillus sp* with the majority (47 clones) being *Lactobacillus animalis*-like organism but there was a single clone of *Lactobacillus murinus*-like organism represented in these samples. *Streptococcus sp* was mainly *Streptococcus sanguinis*-like organism (23 clones) and to a lesser degree *Streptococcus alactolyticus*-like organisms (5 clones).

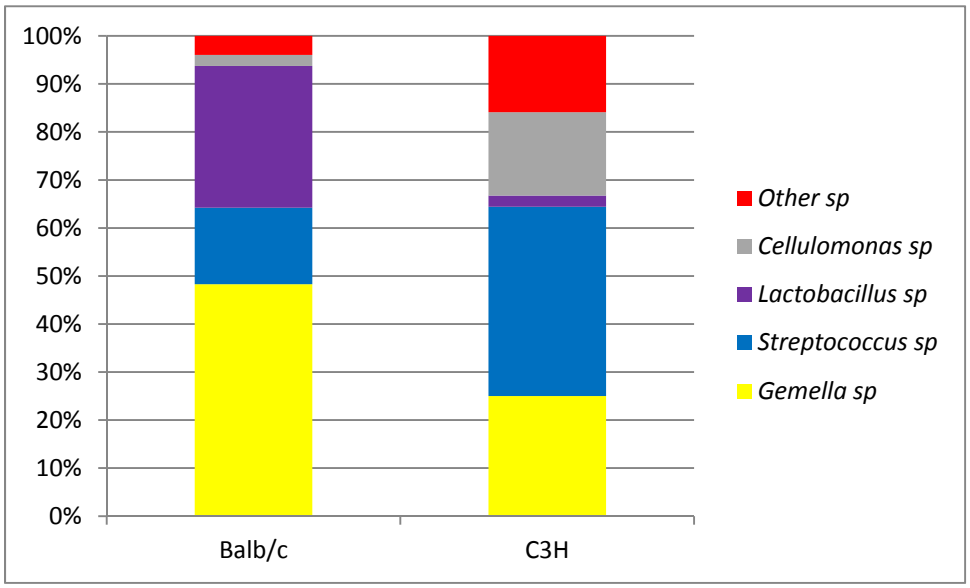

Figure 4.43 The commensal microbiota of C3H/Orl and Balb/cAnNeCrl mice as determined by conventional cloning and sequencing. These data are based on pooled DNA from 3 cages of Balb/c mice and 4 cages of C3H mice (2-6 mice per cage). Each micro-organism is calculated as mean for each cage and expressed as a percentage of the total bacteria present.

4.7.2. Transmission of the commensal microbiota from a SPF Balb/c mouse into a GF C3H mouse

4.7.2.1. Microbial counts in the transmitted Balb/c commensal microbiota

After 1 day of co-caging the previously germ free C3H mice had acquired a commensal microbiota that resembled the animals into whose cages they had been housed. This was the case for total anaerobic (Figure 4.44) and aerobic (Figure 4.45) microbial counts. These levels in GF animals were maintained for the 16 weeks of co-caging and the levels were not significantly different from the Balb/c mice with which they were co-caged ($p>0.05$). The microbial levels within the Balbc/c strain did not differ significantly between start and end of co-caging ($p>0.1$).

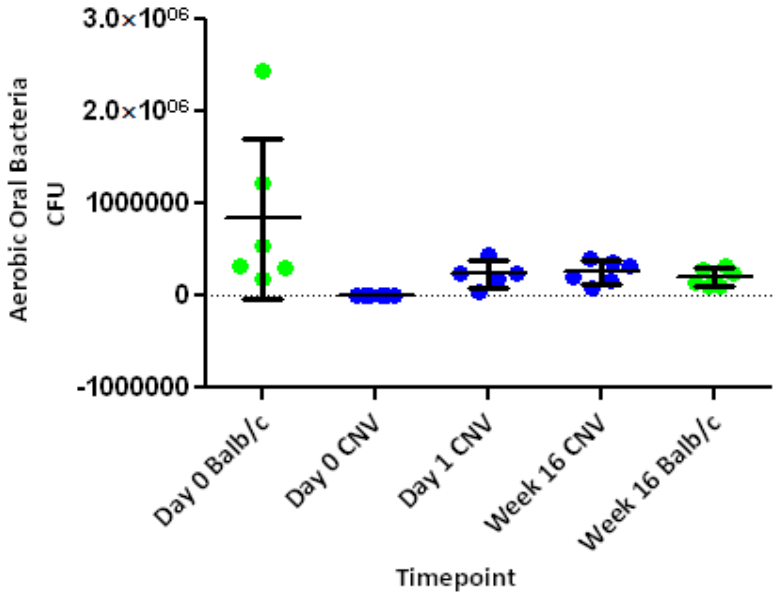

Figure 4.44 Total anaerobic microbial counts for Balb/c mice at baseline (Day 0) and end of experiment (week 16) and C3H conventionalised (CNV) mice (previously germ free) co-caged with them at baseline (Day 0), after 1 day of co-caging (Day 1) and end of experiment (week16). Data expressed as total anaerobic colony forming units (CFU) for each animal with horizontal lines representing mean +/- SD for each group.

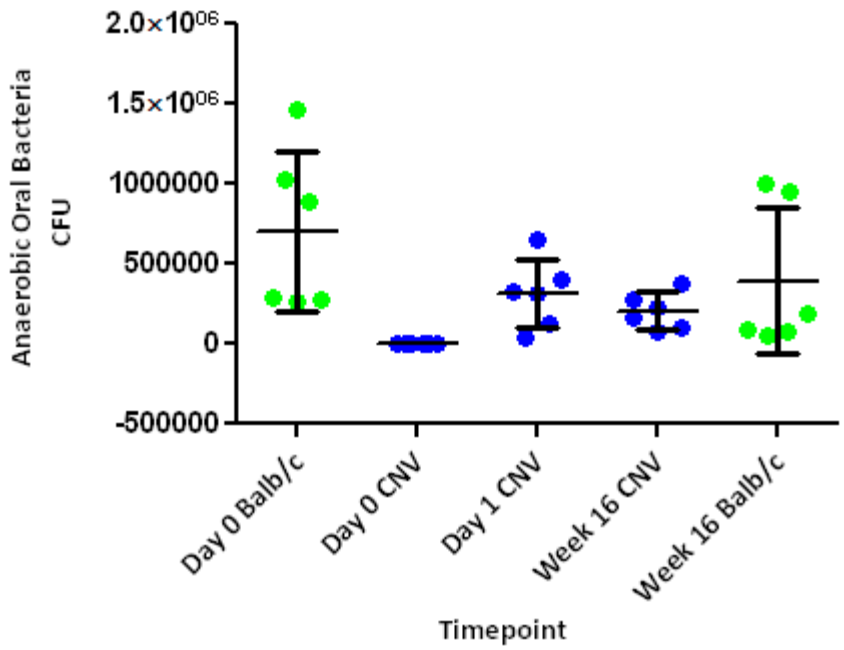

Figure 4.45 Total aerobic microbial counts for Balb/c mice at baseline (Day 0) and end of experiment (week 16) and C3H conventionalised (CNV) mice (previously germ free) co-caged with them at baseline (Day 0), after 1 day of co-caging (Day 1) and end of experiment (week16). Data expressed as total anaerobic colony forming units (CFU) for each animal with horizontal lines representing mean +/- SD for each group.

4.7.2.2. Composition of the transmitted Balb/c commensal microbiota using culture techniques

The major bacterial components of the Balb/c commensal microbiota are completely transmissible into the previously germ free C3H mice (Figure 4.46). The major cultivable aerobic micro-organisms in the Balb/c mice were *Streptococcus sp*, *Lactobacillus sp* and *Escherichia/Shigella sp* and these were detectable in similar proportions in the conventionalised mice when sampled after 16 weeks of co-caging. Similarly, the major cultivable micro-organisms anaerobically were *Streptococcus sp*, *Lactobacillus sp* and *Staphylococcus sp* and these were transferred into the conventionalised mice in similar proportions after co-caging for 16 weeks.

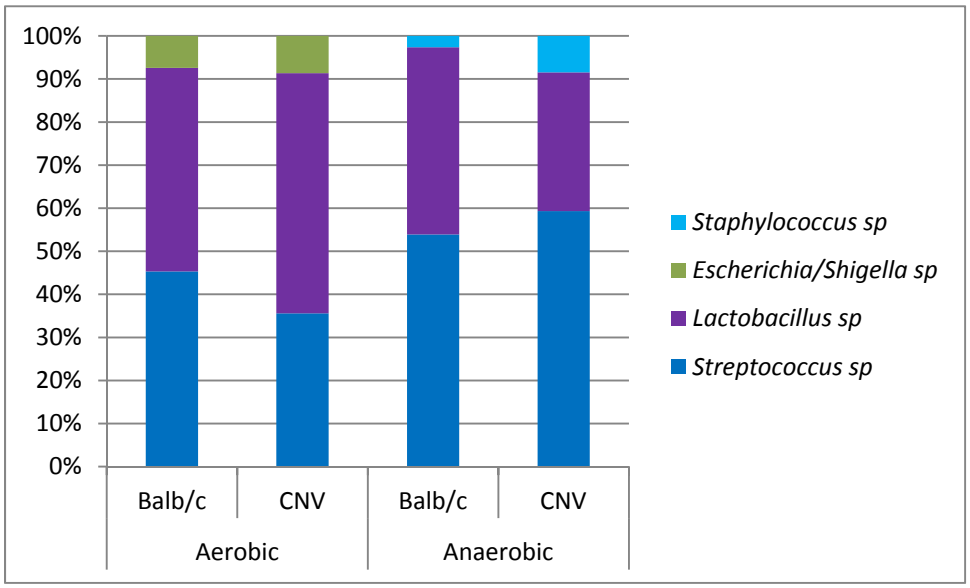

Figure 4.46 Transmission of the cultivable components of the commensal microbiota of the Balb/cAnNeCrl into the conventionalised (previously germ free) C3H/Orl (CNV) after 16 weeks of co-caging. The mean number of each colony was calculated for each animal then the data pooled per cage. Each cage consisted of 2 Balb/c mice and 2 (previously germ free) C3H mice.

4.7.2.3. Composition of the transmitted Balb/c commensal microbiota using non-culture techniques

After 16 weeks of co-caging oral swabs were taken from the Balb/cAnNeCrl mice (SPF) and the conventionalised (previously germ free) C3H/Orl mice (CNV) and the DNA was extracted. The 16S gene was amplified by PCR and amplicons were cloned into *E coli*. Random selection of transformants were sequenced and the sequences interrogated against the BLAST database for identification.

The major components of the commensal microbiota detected by this method were transmitted from the SPF Balb/c mouse into the previously germ free C3H mouse after 16 weeks of co-caging (Figure 4.47). The major components of the Balb/c mice were *Gemella sp*, *Streptococcus sp*, *Lactobacillus sp* and *Cellulomonas sp*. In the Balb/c mice the *Gemella sp* were exclusively *Gemella palacticanis*-like bacteria and the *Cellulomonas sp* exclusively *Cellulomonas persica*-like organism. The *Lactobacillus sp* were mainly *Lactobacillus animalis*-like with very low numbers (1-2 clones) of *Lactobacillus murinus*-like organisms. The *Streptococcus sp* were mainly *Streptococcus sanguinis*-like organisms with lower numbers (5 clones) of *Streptococcus alactolyticus*-like bacteria.

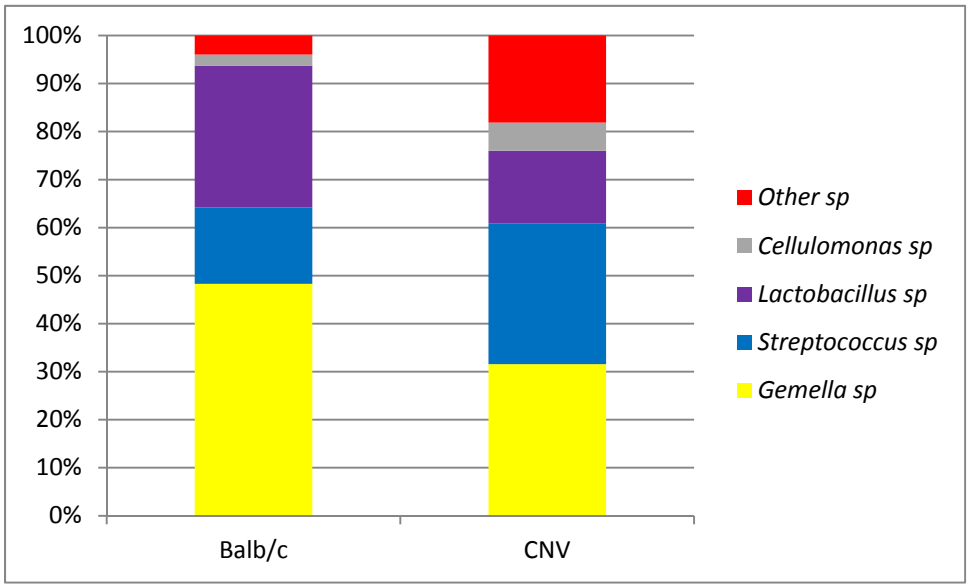

Figure 4.47 The composition of the commensal microbiota of Balb/cAnNeCrl mice (Balb/c) and transmission into a C3H/Orl germ free mouse (CNV) after 16 weeks of co-caging. Data is based on the pooled DNA from 2 mixed cages made up of 2 Balb/c mice and 2 C3H mice. The mean number of transformants for each micro-organism is expressed as a percentage of the total number of sequences.

More detailed analysis of the major components of the Balb/c, conventionalised and C3H highlights some differences (Figure 4.48). The transfer of the oral commensal microbiota of the Balb/c mice was complete with respect to the major species and strains as identified using a 'best hit' approach on the NCBI BLAST database. In these two groups of animals (Balb/c and CNV) *Streptococcus sanguinis*-like and *Streptococcus alactolyticus*-like organisms are present in similar numbers and are the only strains of *Streptococcus sp* detected. Compared to the C3H mice (the background of the previously germ free CNV mice) there appears to be greater diversity within the *Streptococcus sp* with lower numbers of *S. salivarius*, *S. intermedius* and *S. equi*-like bacteria. In addition, in these C3H mice the dominant strain of *Streptococcus sp* is *S. alactolyticus*-like organisms rather than *S. sanguinis*-like organisms which predominate in the Balb/c and conventionalised mice. The other major difference between C3H and Balb/c in terms of the major components is the presence of *Cellulomonas sp* in the Balb/c (and transferred into the CNV) which are not detectable in the C3H mice in this experiment; predominantly *Cellulomonas persica* like bacteria.

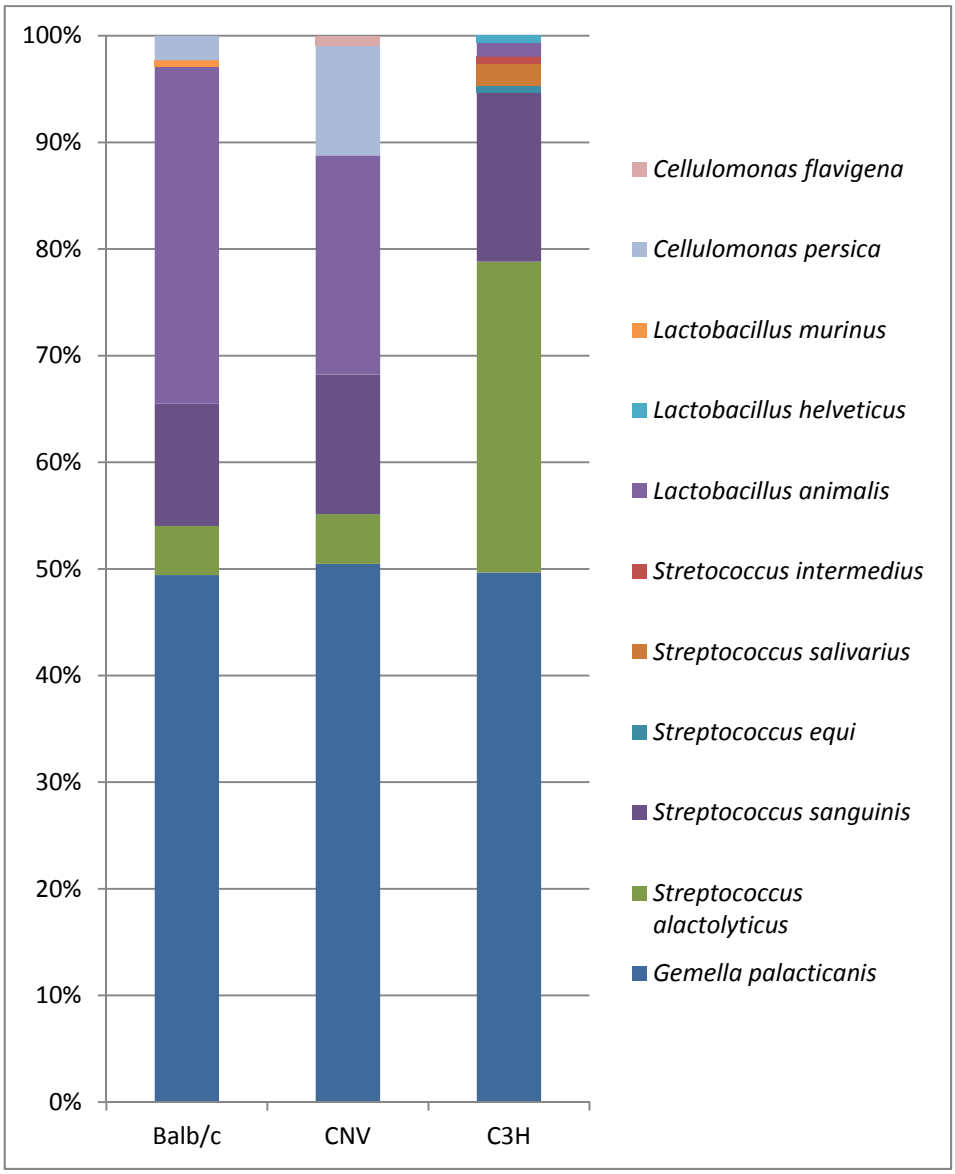

Figure 4.48 The composition of the major components of the oral commensal microbiota of Balb/c mice which were co-caged with previously germ free C3H mice (CNV) for 16 weeks and comparison with the wild type commensal microbiota of the SPF C3H mice (background strain for the CNV mice). Analysis based on pooled DNA from oral swabs from cages of 3 cages of mixed Balb/c and CNV mice (2 Balb/c per cage and 2-3 CNV) and pooled DNA from 2 cages of C3H mice (2-6 mice per cage). The 16s rRNA gene was amplified and cloned into *E. coli* with random transformants selected and sequenced with identification based on the NCBI BLAST database.

Analysis of the 'other' components of the oral commensal microbiota of the Balb/c mice and the co-caged CNV mice showed differences (Figure 4.49). The conventionalised mice had a disproportionately large number (13 clones) of *Staphylococcus aureus*-like organisms detectable in the 'other' group of micro-organisms. These were not present in the Balb/c mice into whose cages the CNV mice were housed. The source of this micro-organism is likely to be from the skin of the operator and due to contamination of the samples during processing. Otherwise, the components of this 'other' group were highly diverse and present in very low numbers (1-2 clones). They were different between the two sets of mice (Balb/c and CNV) with no overlap. These differences represent a 'tail of diversity' of very low number and highly varied micro-organisms the extent and significance of which cannot be determined with these investigations (see section 5.2.3. and 6.1. for discussion of the implications of this).

So it would appear that there are some minor differences between different strains of SPF mice (Balb/c compared to C3H) but that the commensal microbiota of the Balb/c mice is transmissable to the previously germ free C3H mice. The significance of this is that although the mouse strain may influence the composition of the oral commensal microbiota it does not seem to affect its transmission into a different strain. The microbiota is the determining factor rather than the strain of mouse into which it is transmitted.

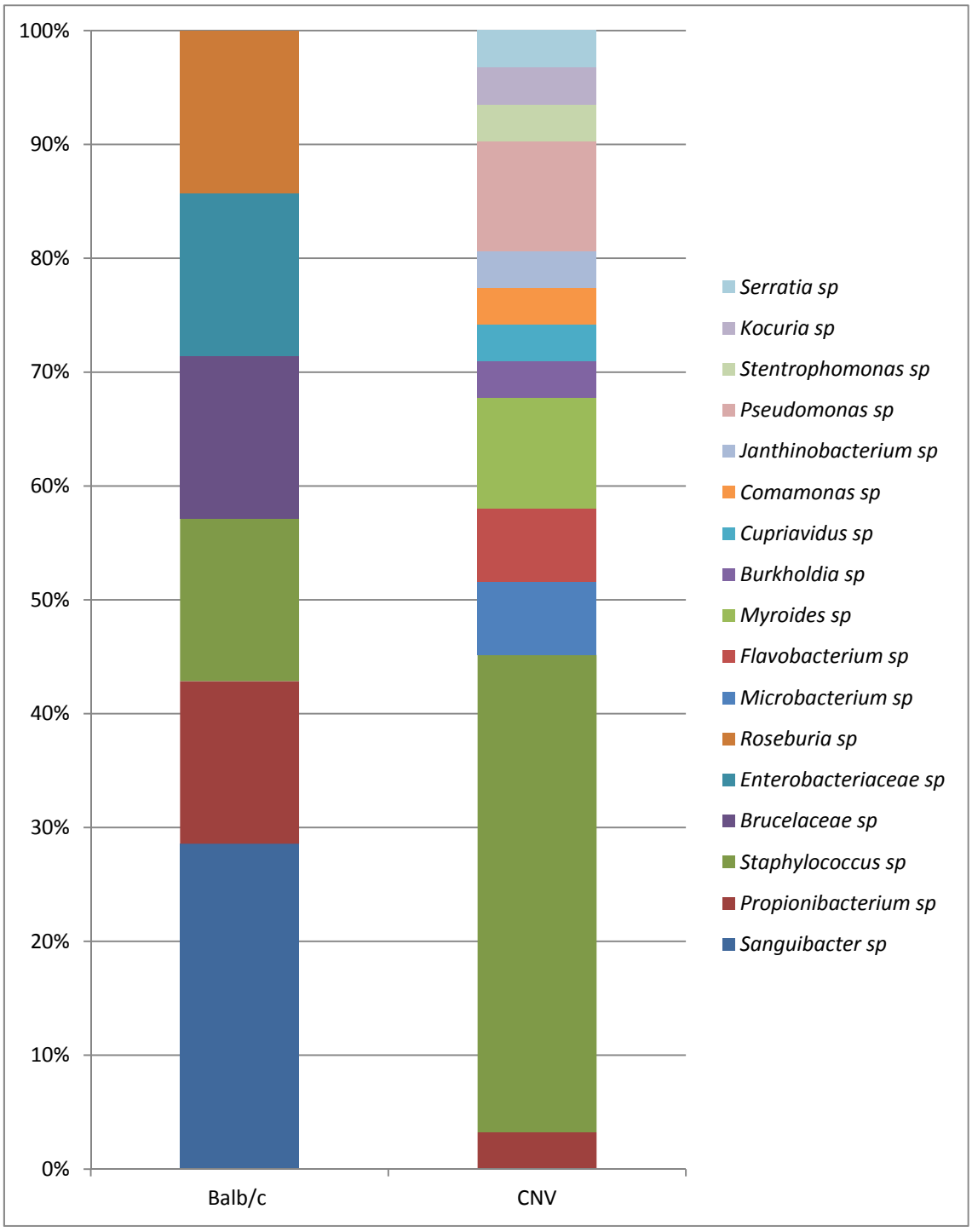

Figure 4.49 The compostion of the 'other' group of micro-organisms in the oral commensal microbiota of Balb/c mice and the previously germ free C3H mice (CNV) that were co-caged with them. Each micro-organism is expressed as its proportion of the total component micro-organisms in the 'other' group.

4.7.2.4. Alveolar bone loss

Despite differences in mouse strain the same commensal microbiota appears to be transmitted from the Balb/c SPF mouse to the (previously germ free) C3H CNV mouse. At the end of the 16 weeks of co-caging bone levels are not significantly different ($p=0.3532$) (Figure 4.50). However, the alveolar bone levels of age matched C3H SPF mice was significantly lower ($p<0.0001$) i.e. greater bone loss. This would suggest the slight differences in the oral commensal microbiota of the Balb/c and C3H SPF mice has an effect on the degree of periodontal bone loss, the implication being that the microbiota that had developed in the SPF C3H mouse is more pathogenic than the microbiota that had developed in the Balb/c mouse. Transmission of this less pathogenic microbiota into the previously germ free C3H mouse did not develop the same level of alveolar bone loss as the wild type SPF C3H mouse. (Intra-operator reliability see Appendix 7.6).

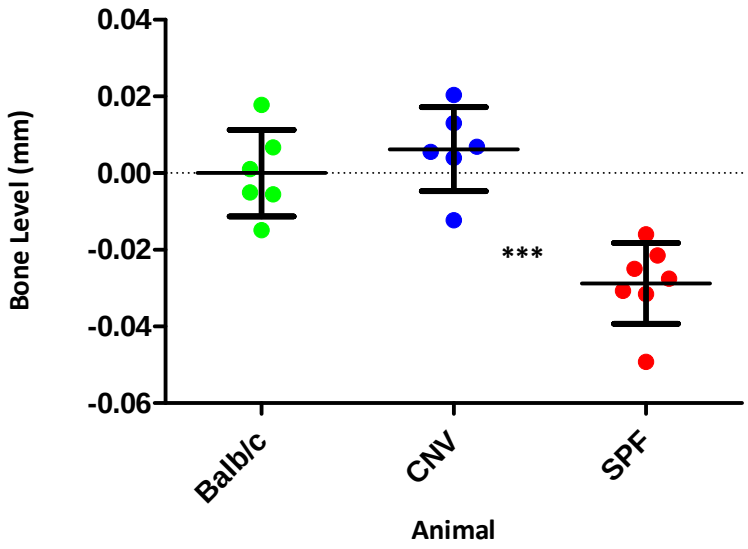

Figure 4.50 Periodontal bone levels for Balb/c SPF mice (green dots) co-caged with previously germ free C3H mice (CNV blue dots) assessed after 16 weeks of co-caging and comparison with age matched C3H SPF mice (SPF red dots). All data is shown for individual mice with the horizontal lines representing the mean bone level +/- SD. All measurements are relative to the Balb/c wild type animal negative values indicating alveolar bone loss. *** represents statistically significant difference $p<0.0001$. (Intra-operator reliability see Appendix 7.6).

4.8. Influence of Mouse Genotype on Commensal Microbiota

The commensal microbiota of one strain of an SPF mouse; Balb/cAnNeCrl is completely transmissible to a different strain of a previously GF mouse; C3H/Orl. The commensal microbiota is very similar in terms of aerobic and anaerobic microbial counts and also in terms of the micro-organisms that make up the commensal microbiota. This raises the question as to whether the mouse genotype within a particular strain has an influence on the nature of the commensal microbiota.

To test this hypothesis we used 2 different groups of Balb/c mice. The wild type Balb/c and a transgenic mutant CXCR2 knockout mouse in the same Balb/c background. This latter mouse was deficient in the receptors for the ligands of the mouse homologue of Interleukin 8 (IL8) which is an important cytokine in the host inflammatory response. We compared the commensal microbiota of age and sex matched wild type and knockout mice using culture and non culture techniques previously described.

4.8.1. Defining the commensal microbiota of the CXCR2$^{-/-}$ mouse

4.8.1.1. Microbial counts in the commensal microbiota of the CXCR2$^{-/-}$ mouse

The knockout CXCR2ko mice had statistically significantly increased levels of micro-organisms cultured anaerobically (Figure 4.51) and aerobically (Figure 4.52) when compared to wild type Balb/c mice. This was maintained for the 16 weeks of the experiment.

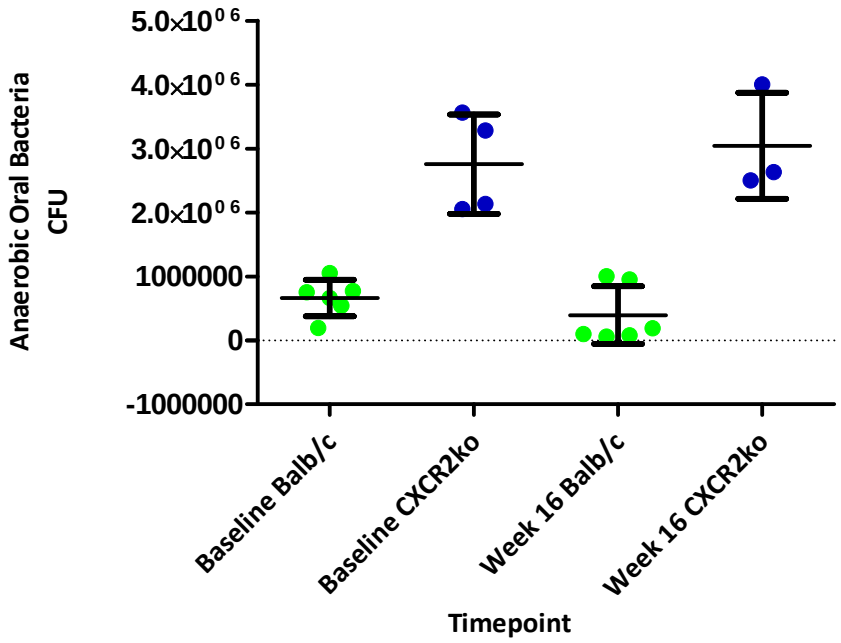

Figure 4.51 Total anaerobic microbial counts for wild type Balb/c and knockout CXCR2ko mice at baseline and after 16 weeks. The knockout mice have significantly higher numbers of anaerobic micro-organisms than the wild type. Data expressed as total anaerobic counts for each animal with horizontal lines representing mean values +/- SD.

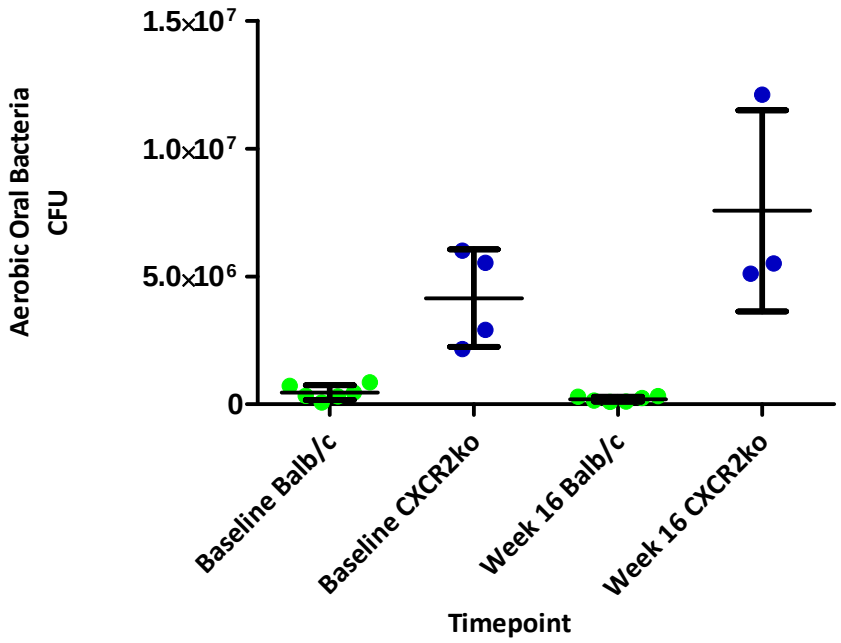

Figure 4.52 Total aerobic microbial counts for wild type Balb/c and knockout CXCR2ko mice at baseline and after 16 weeks. The knockout mice have significantly higher numbers of aerobic micro-organisms than the wild type. Data expressed as total anaerobic counts for each animal with horizontal lines representing mean values +/- SD.

4.8.1.2. Composition of the commensal microbiota of the CXCR2$^{-/-}$ mouse using non-culture techniques

Oral swabs were taken from SPF Balb/cAnNeCrl mice and SPF CXCR2-/- mice and the DNA was extracted. The 16S gene was amplified by PCR and amplicons were cloned into plasmid inserts from *E coli*. Random selection of transformants were sequenced and the sequences interrogated against the BLAST database for identification.

Based sequence analyses of the major components of the oral commensal microbiota of the Balb/c mice and CXCR2-/- differences were detectable (Figure 4.53). In both sets of mice the major micro-organisms were *Gemella sp*, *Streptococcus sp* and *Lactobacillus sp* with *Cellulomonas sp* only detectable in the Balb/c mice.

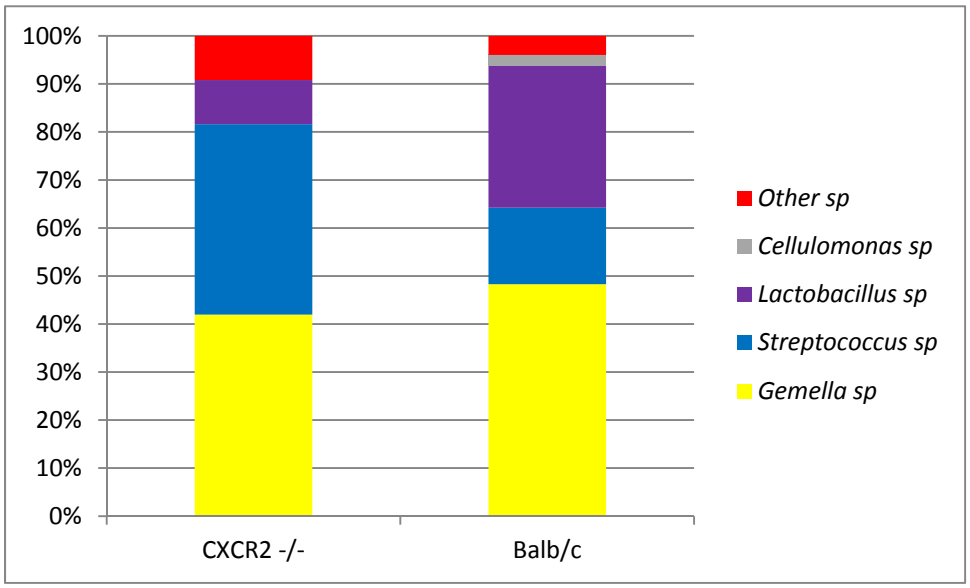

Figure 4.53 The commensal microbiota of CXCR2$^{-/-}$ and Balb/cAnNeCrl mice as determined by conventional cloning and sequencing. These data are based on pooled DNA from 3 cages of Balb/c mice and 2 cages of CXCR2$^{-/-}$ mice (2-6 mice per cage). Each micro-organism is calculated as mean for each group and expressed as a percentage of the total bacteria present. Samples taken after 14 days of co-caging.

More detailed investigation (Figure 4.54) showed that the *Gemella sp* is exclusively *Gemella palacticanis*-like organism, *Streptococcus sp* is mainly a *Streptococcus sanguinis*-like bacteria in the Balb/c mice whereas it is largely a *Streptococcus alactolyticus*-like organism in the CXCR2$^{-/-}$. The *Lactobacillus sp* is mainly *Lactobacillus animalis*-like bacteria in both sets of mice and the *Cellulomonas sp* varies between the two mouse strains being *Cellulomonas persica*-like in the Balb/c and *Cellulomonas hominis*-like bacteria in the CXCR2$^{-/-}$.

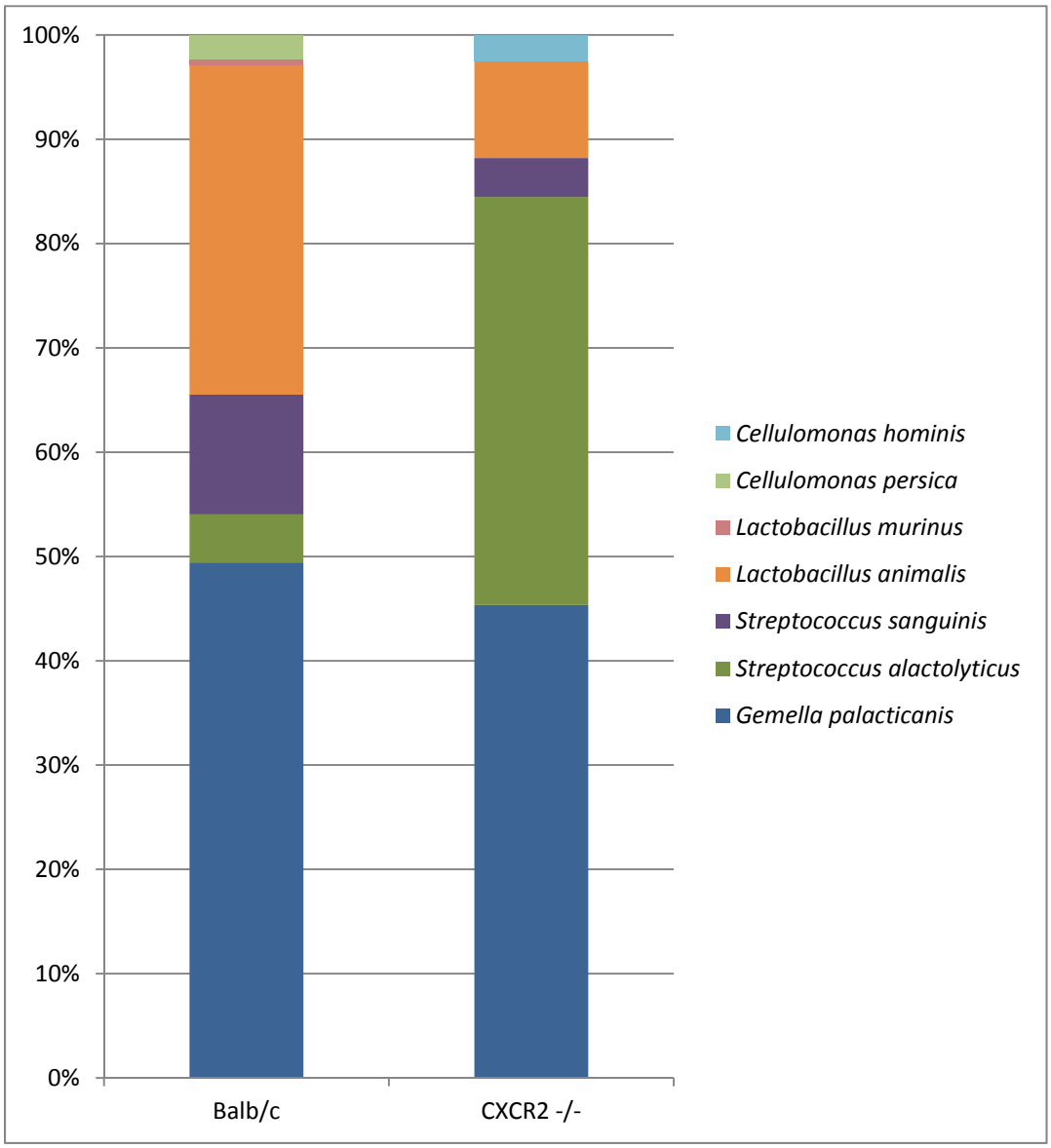

Figure 4.54 The composition of the major components of the oral commensal microbiota of wild type Balb/c mice and the CXCR2$^{-/-}$ knockout strain. Analysis based on pooled DNA from oral swabs of 3 cages of Balb/c and 2 cages of CXCR2$^{-/-}$(2-4 mice per cage). The 16s rRNA gene was amplified and cloned into *E. coli* with random transformants selected and sequenced with identification based on the NCBI BLAST database. Samples taken after 14 days of co-caging.

4.8.1.3. Alveolar bone Levels

The knockout mice (CXCR2ko) had a different oral commensal microbiota when compared to wild type (Balb/c) both in terms of numbers of micro-organisms and the types of micro-organisms that constitute this commensal microbiota. This difference was manifest in significantly lower levels of periodontal bone in the knockout mice (Figure 4.55). (Intra-operator reliability see Appendix 7.7).

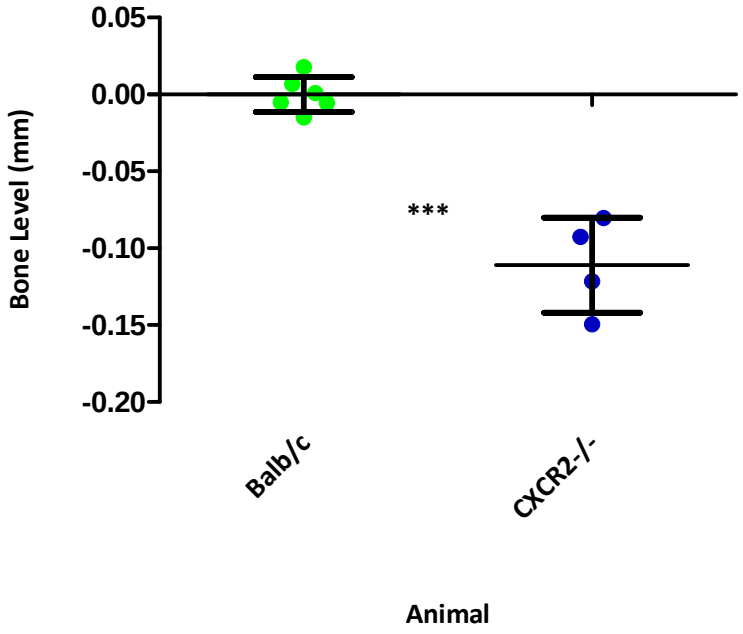

Figure 4.55 Periodontal bone levels for Balb/c SPF mice and CXCR2$^{-/-}$ mice assessed after 16 weeks of co-caging. All data are shown for individual mice with the horizontal lines representing the mean bone level +/- SD. All measurements are relative to the Balb/c wild type animal. *** represents significance P<0.0001. (Intra-operator reliability see Appendix 7.7).

4.8.2. Transmissibilty of the commensal microbiota from a SPF CXCR2$^{-/-}$ mouse into a GF C3H mouse

The oral commensal microbiota of the knockout mice (CXCR2$^{-/-}$) was different from that of the wild type (Balb/c) primarily in terms of numbers of micro-organisms present and this led to increased periodontal bone loss. It is worth remembering that the knockout mouse has compromised host inflammatory response through loss of receptors for IL8. This could account for the increased microbial load within the commensal microbiota and possibly compositional differences. This leads to the question as to whether this commensal microbiota (another form of altered flora; altered by host geneotype rather than *P. gingivalis* challenge) is transmissible to a germ free animal and the effect of the resultant effect on periodontal bone levels.

Another co-caging experiment was performed whereby SPF Balb/c wild type and SPF CXCR2$^{-/-}$ (genotype altered flora) were co-caged with germ free C3H mice. Transmission of the commensal microbiota was monitored by culture and non-culture methods and periodontal bone loss assessed after 16 weeks of co-caging.

4.8.2.1. Microbial counts in the transmitted microbiota

Transmission of the commensal microbiota from wild type Balb/cAnNeCrl mice to germ free C3H/Orl mice occured quickly and completely (see section 4.7). This led to similar levels of periodontal bone after 16 weeks of co-caging. The genotype-altered microbiota of the CXCR2$^{-/-}$ mice did not transfer into a GF C3H mouse as readily or completely. The microbial counts both anaerobically (Figure 4.56) and aerobically (Figure 4.57) were significantly lower in the co-caged C3H CNV (previously germ free) mice than for the knockout mice into whose cages they were housed. This remained throughout the period of co-caging (16 weeks).

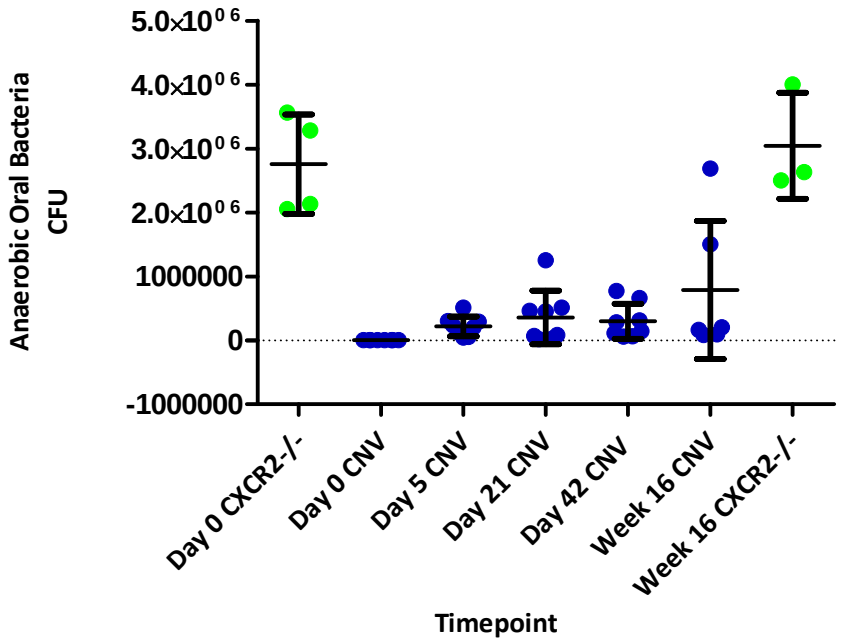

Figure 4.56 Anaerobic microbial counts for conventionalised (CNV) previously germ free C3H mice (blue points) co-caged with CXCR2-/- Balb/c mice (green points) at time points after co-caging. Data is presented for each mouse with horizontal lines representing mean values +/- SD for each group. At all time points the levels of anaerobic micro-organisms are significantly lower than for CXCR2$^{-/-}$ mice ($p<0.05$).

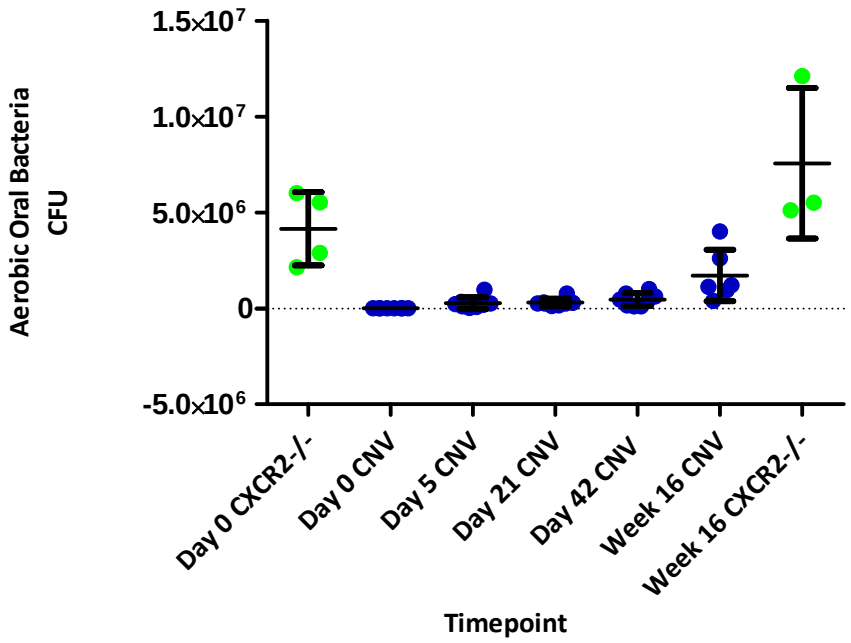

Figure 4.57 Aerobic microbial counts for conventionalised (CNV) previously germ free C3H mice (blue points) co-caged with CXCR2$^{-/-}$ Balb/c (green points) mice at time points after co-caging. Data is presented for each mouse with horizontal lines representing mean values +/- SD for each group. At all time points the levels of aerobic micro-organisms are significantly lower than for CXCR2$^{-/-}$ mice ($p<0.05$).

4.8.2.2. Composition of the commensal microbiota of the transmitted CXCR2-/- microbiota into GF C3H mice using non-culture techniques

In terms of the major components of the commensal microbiota of the CXCR2$^{-/-}$ mouse there appeared to be complete transmission into the previously germ free conventionalised (CNV) C3H mice after 16 weeks of co-caging (Figure 4.58).

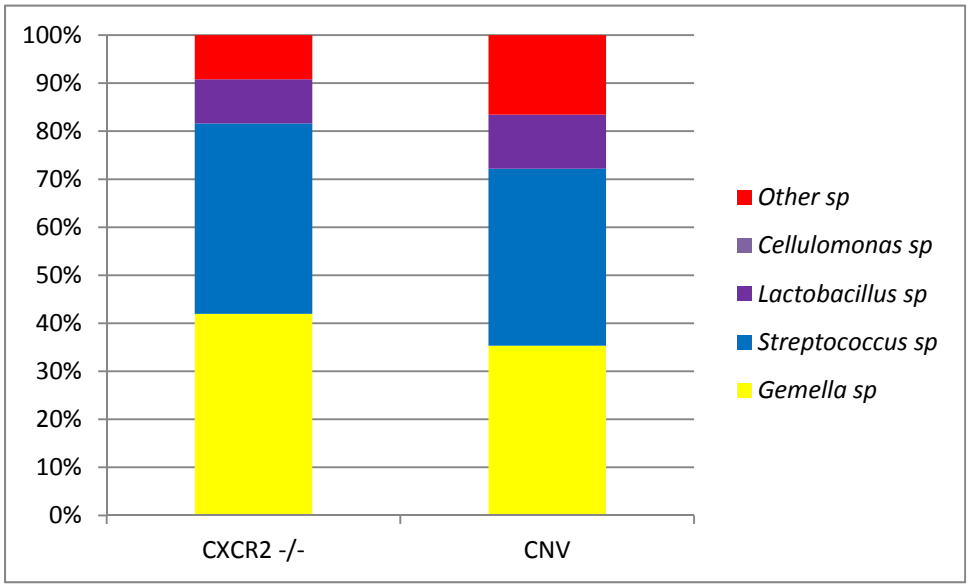

Figure 4.58 The composition of the commensal microbiota of the CXCR2$^{-/-}$ mice and its transmission into previously germ free conventionalised C3H/Orl (CNV) mice following oral sampling and extraction of the DNA. The 16S gene is then amplified and cloned into *E coli*. Random transformants are selected and sequenced for bacterial identification. Samples taken after 14 days of co-caging.

More detailed analysis of the major components of the oral commensal microbiota showed there were differences in the microbiota that was transferred from the CXCR2$^{-/-}$ into the conventionalised (previously germ free) CNV mice (Figure 4.59). The *Gemella sp* was exclusively *Gemella palacticanis*-like in both knockout and conventionalised mice. The *Streptococcus sp* consists of *Streptococcus alactolyticus*-like and *Streptococcus sanguinis*-like bacteria in both sets of mice with a preponderance of *S. alactolyticus*-like organisms in the knockout mice compared to the CNV mice. In addition, there was greater diversity in the CNV mice which received the commensal microbiota from the knockout mice, these included low numbers (1-3 clones) of *S. equi*-like, *S. parasanguinis*-like, *S. orisuis*-like and *S. mutans*-like bacteria in the CNV mice but not detected in the knockout mice. There were 2 major differences between the 2 sets of mice. Firstly, the strain difference; the knockout mice were in a Balb/c background whilst the conventionalised mice were in a C3H/Orl background. Secondly, the C3H mice have an intact immune system which is lacking in the CXCR2$^{-/-}$ mice. Both these differences may account for this difference in the transmitted components of the oral commensal microbiota.

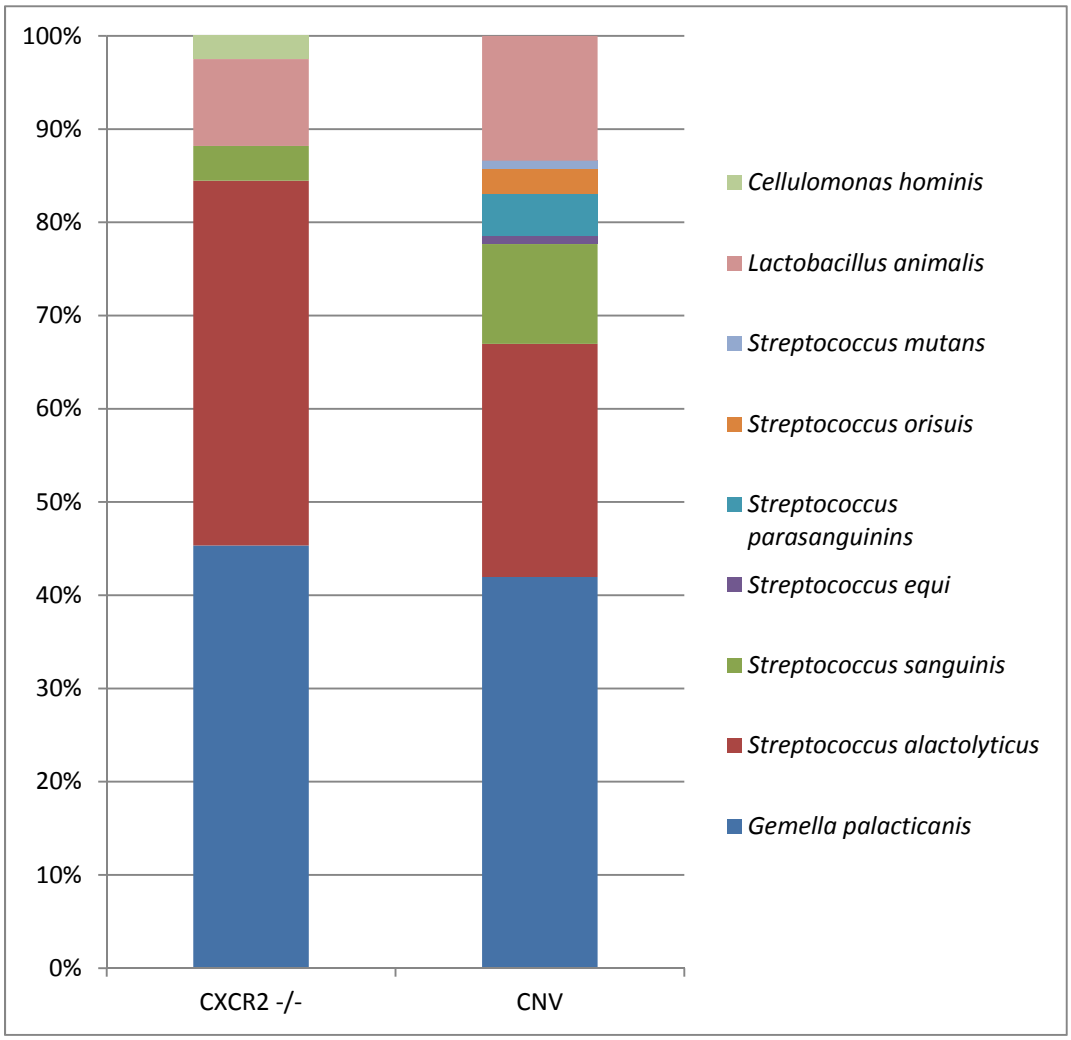

Figure 4.59 The composition of the major components of the oral commensal microbiota of CXCR2$^{-/-}$ knockout strain of Balb/c mice and its transmission into previously germ free conventionalised (CNV) C3H mice . Analysis based on pooled DNA from oral swabs from mixed cages of CXCR2$^{-/-}$ and previously germ free conventionalised (CNV) mice (2-4 mice per cage). The 16s rRNA gene was amplified and cloned into *E. coli* with random transformants selected and the plasmid insert DNA sequenced with identification based on the NCBI BLAST database.

In addition there were significant differences in the composition of the micro-organisms in the 'other' group (Figure 4.60) between the host CXCR2$^{-/-}$ and the conventionalised C3H mice. Again the 2 groups shared a number of common components in this group (*Sanguibacter sp*, *Acinetobacter sp*, *Pseudomonas sp*, *Erysipelotrichaceae sp* and *Enterobacteria sp*) but the conventionalised had further diversity with 6 unique species of micro-organisms (*Leuconostoc sp*, *Paenbacillus sp*, *Gammaproteobacteria sp*, *Actinomycetales sp*, *Janthinobacterium sp* and *Microbacterium sp*). Again this may be accounted for by mouse strain differences or by different abilities to mount inmmune/inflammatory responses in the two strains of mice.

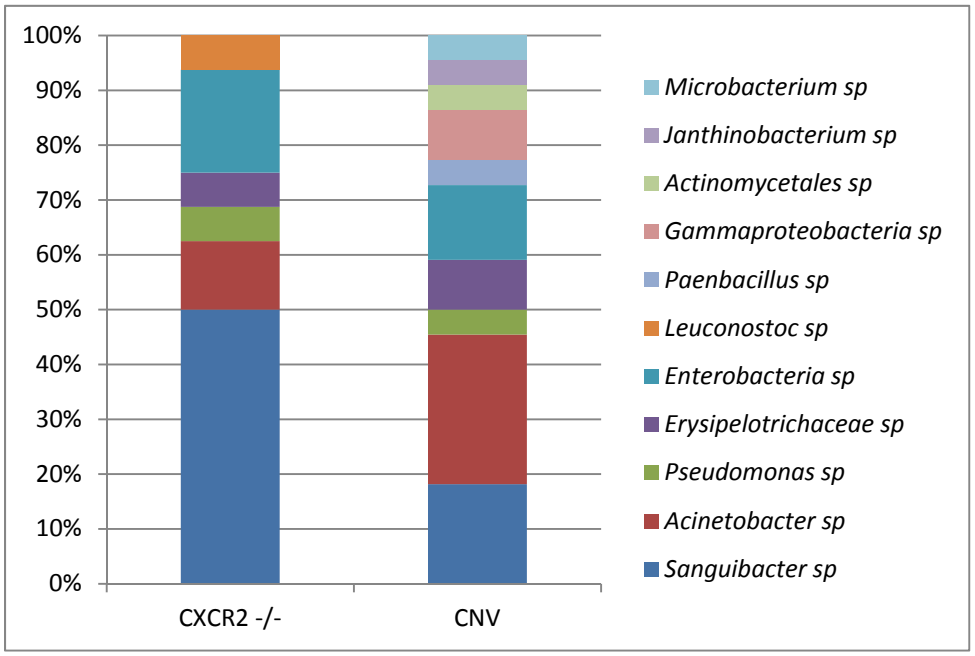

Figure 4.60 The compostion of the 'other' group of micro-organisms in the oral commensal microbiota of CXCR2$^{-/-}$ and the previously germ free C3H mice (CNV) that were co-caged with them. Each micro-organism is expressed as its proportion of the total component micro-organisms in the 'other' group.

4.8.2.3. Alveolar bone Loss

The transmission of the commensal microbiota from a SPF Balb/c mouse to a CNV C3H mouse is complete and does not lead to any significant differences in levels of periodontal bone (See Section 4.7). The transmission of the genotype altered microbiota of a SPF CXCR2$^{-/-}$ mouse (Balb/c background) into a GF C3H/Orl mouse is incomplete in terms of microbial load and composition and does not lead to increased periodontal bone loss. Indeed the periodontal bone levels of the previously germ free C3H mice were significantly higher (less bone loss) than those animals housed with Balb/c controls (Figure 4.61). This may be due to the development of the biofilm in the CXCR2$^{-/-}$ mouse progressing largely unchecked due to impaired neutrophil function and migration. Under these conditions the biofilm may not need to be as virulent to thrive and following transmission into a more immune-competent host (the C3H mouse) it may lack some of the strategies to overcome these. The result is reduced bone loss upon transmission. (Intra-operator reliability see Appendix 7.8).

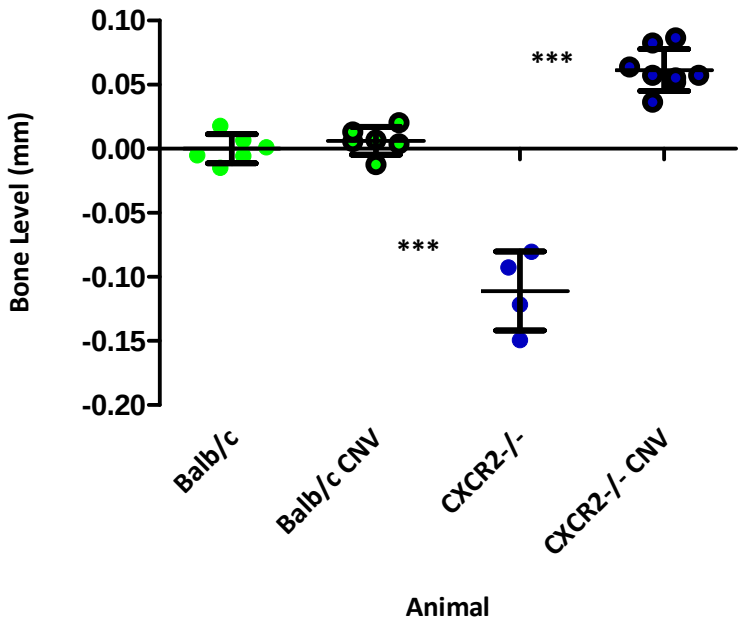

Figure 4.61 Bone levels for C3H germ free mice (open points) when co caged with Balb/c wild type (green points) and Balb/c CXCR2$^{-/-}$ (blue points) mice. Data is presented for each animal with horizontal lines representing means +/- SD. Bone loss relative to Balb/c wild type controls in represented as negative bone levels. *** represents significance at $p<0.0001$ relative to Balb/c controls. Analysis performed after 16 weeks of co-caging. (Intra-operator reliability see Appendix 7.8).

The significance of these finding presented inthis section will be discussed in the next chapter (Section 5) and the implications for further research (Section 6).

4.9 Reliability and sensitivity of alveolar bone measurement

The two sets of measurements used to explore the reproducibility of repeated measurements of alveolar bone loss are presented as Pearson correlation coefficients for each group of animals in each experiment in appendix seven. Briefly, in all groups of animals in each experiment the correlation coefficient was greater than 0.6 ($p<0.09$). However, in most groups the correlation coefficient exceeded 0.8 ($p<0.05$). Generally, the smaller the number of animals in each group and the smaller the measurements of alveolar bone loss the less reliable the data became.

Chapter 5

Discussion

5. Discussion

5.1. The commensal microbiota

5.1.1. The commensal microbiota of specific pathogen free (SPF) mice

The result of the experiments presented in this thesis show that the commensal microbiota of the experimental mice is a constant and stable feature of their overall microbiome. In establishing the genetically identical background for both germ free (GF) and specific pathogen free (SPF) mice we selected a 'simple' cultivable commensal oral microbiota from the different batches of SPF mice delivered. The transmission of this selected microbiota into the GF mice by co-caging with SPF mice was complete. Subsequent analysis of this microbiota by non-culture techniques confirmed its relative simplicity but was able to highlight subtle differences in the specific strains of the major micro-organisms, especially *Streptococcus sp*. In addition, it demonstrated a highly diverse range of micro-organisms that are present in very low numbers suggesting a level of complexity to the commensal oral microbiota that would benefit from further investigation.

The cultural data defining the commensal oral microbiota in these studies largely corresponds with earlier investigations defining the mouse oral commensal microbiota by other groups. Early studies using solely culture techniques to isolate specific micro-organisms which were then characterised and identified based on biochemical and structural differences concur that a relatively simple microbiota is dominant[424]. This study in Balb/c mice found the predominant micro-organisms were *Lactobacillus murinus* (38%), *Staphylococcus aureus* (37%), *Streptococcus faecalis* (8%), *Staphylococcus sciuri* (4%) and *Escherichia coli* (3%). Another study investigated the oral commensal microbiota using colony immunoblotting of cultured isolates and specific blood and saliva immunoglobulin responses in Balb/c mice. Once again a simple microbiota was discovered[425]. *Lactobacillus murinus*, *Enterococcus faecalis*, *Streptococcus oralis* and *Staphylococcus epidermis* were determined to be the predominant micro-organisms.

The use of non-culture techniques has also been used to demonstrate bacterial diversity in the mouse oral commensal microbiota[416]. These authors used next generation high throughput sequencing to investigate the oral commensal microbiota in wild type and TLR2-deficient mice in a C57/BL6 background. They estimated the diversity in the microbiome to be around 200 species but importantly a simple commensal microbiota consisting of 2 major phyla was predominant (>90%). *Firmicutes* and *Proteobacteria* were the dominant phyla and of these 88% were represented by 10 major species. There was large variation between individual mice but the major species were *Streptococcus sp*, *Staphylococcus sp*, *Lactobacillus sp* and *Enterococcus sp*. Interestingly this study failed to detect *Gemella sp* but there was a high proportion of *Staphylococcus lentus*, *S. sciuri*, *S. xylosus* and *S. cohnii* which might be an area in which the errors inherent in high throughput sequencing may have manifested as mis-identification of micro-organisms.

Defining the commensal microbiota of the C3H mouse in this thesis has been paramount in understanding the changes that occur within it in response to periodontal pathogens and the concept of dysbiosis as a driving force in the pathogenesis of periodontal disease. This is the first work to extensively define the oral commensal microbiota in this C3H mouse model[198].

In these studies we have deliberately selected a simple oral microbiota to optimise the sensitivity of any variations and to be able to detect these variations in a group of micro-organisms that we can reliably detect. It is worth noting that that even in animals of different strains supplied for transmission experiments (Balb/c) that the microbiota upon delivery was still relatively simple (see section 4.7). Even in the batch of C3H mice with the 'more complex' oral microbiota, which it was decided not to use as the 'normal' microflora, the composition was still relatively simple (see section 3.2). There were only 8 species of micro-organisms detectable by culture techniques.

The relatively simple oral microbiota of the mouse is in contrast to that in the human. Current data from the Human Oral Microbiome Database (HOMD, http://www.homd.org/) puts the total bacterial species in the human oral cavity at around 700, of which only 49% have been officially named[112]. This number may in reality be closer to 1000 different bacterial species with huge variations between healthy individuals and different disease states[426]. The dominant phyla are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochetes* and *Fusebacteria* which comprise over 96% of the total oral bacteria. It has been estimated that only 50% of the bacteria present in the oral cavity have been isolated and cultivated and this represents around 280 species[112]. There are huge challenges in trying to culture species such as *Treponema sp* of which it has been estimated that only 25% have been isolated and cultured from those present in the oral commensal microbiota. The predominant cultivable bacterial species found in the majority of individuals are *Streptococcus sp* and *Veillonella sp* and these colonise the majority of sites within the oral cavity[160].

Despite the relative simplicity of the mouse oral microbiome there appears to be a similar range and diversity with the dominant phyla being *Proteobacteria* and *Firmicutes*. There is similar diversity in the range of Gram-positive and Gram-negative species and in these respects there are similarities to the human oral microbiome.

Differences might be explained by environment; humans and mice (especially laboratory mice) inhabit very different ecological niches. There are differences in the immune and inflammatory responses to bacterial colonisation of the oral cavity, especially in periodontal models, in the mouse the inflammatory infiltrate is dominated by lymphocytes compared to the initial inflammatory response in humans being neutrophil dominated[252]. Transmission patterns may be different, mice engage in coprophagia which offers an additional means of transmission of the commensal microbiota and also introduces the possibility of a more gut-like microbiome.

5.1.2. The stability of the oral commensal microbiota

In addition to defining the oral commensal microbiota in the C3H mouse these studies have shown that this simple microbiota is a stable community. The cultural data supports the maintenance of this microbiota at quantitative and qualitative levels in different ages of mice sampled at different time points. The non-cultural data compliments this with 3 major species predominating; *Gemella sp*, *Lactobacillus sp* and *Streptococcus sp*. These are important considerations when we examine the pattern of alveolar bone loss in an aging mouse.

The data presented in this thesis with respect to alveolar bone loss in the aging mouse is largely complimentary. Alveolar bone loss occurs in both SPF and GF mice at a slow but steady rate up until around 9 months of age when accelerated bone loss is observed. At all time points the alveolar bone of comparatively aged GF mice is lower than SPF. The data presented in this thesis shows that this bone loss occurs despite a stable and consistent oral microbiota. The implications being that there is some other function of aging that leads to increased periodontal bone loss separate from changes in the oral microbiota. This is consistent with recent studies that have highlight a role of the neutrophil in the aging process and especially its migration into the tissues via endothelial integrins LFA1 and the action of its inhibitor Del1[335].

5.1.3. Differences between culture and non-culture techniques – The *Gemella* effect

One of the major issues in defining the oral commensal microbiota has been the variation in one of the major component micro-organisms detected by each method, namely the detection of *Gemella sp* by non-culture techniques but not by culture techniques. More specifically sequence alignment and interrogation of the NCBI BLAST database show that this is almost exclusively *Gemella palacticanis*. *G. palacticanis* is a Gram-positive coccus that tends to occur in pairs, clusters or short chains. It is facultatively anaerobic forming non-pigmenting and non-haemolytic, low convex, circular, entire colonies on blood agar. This micro-organism was first isolated and described in a sample from an oral abscess of a dog[423]. Its taxonomic lineage is in the family of Staphylococcaceae.

The failure to detect this micro-organism by culture means could be due to fastidiousness. Although it grows on blood agar its preferred media is Trypticase soy agar supplemented with blood and the environment is enriched with CO_2. The growth conditions supplied by blood agar may not be right for its growth or it may require metabolites from other micro-organisms to thrive. Consequently, colonies do not develop to allow their isolation and subculture for identification. Equally, relying on morphological differences to determine different bacteria is open to a degree of interpretation and error and therefore it is possible that this organism was not differentiated on the basis of morphology (Figure 5.1) and so not isolated and processed for identification.

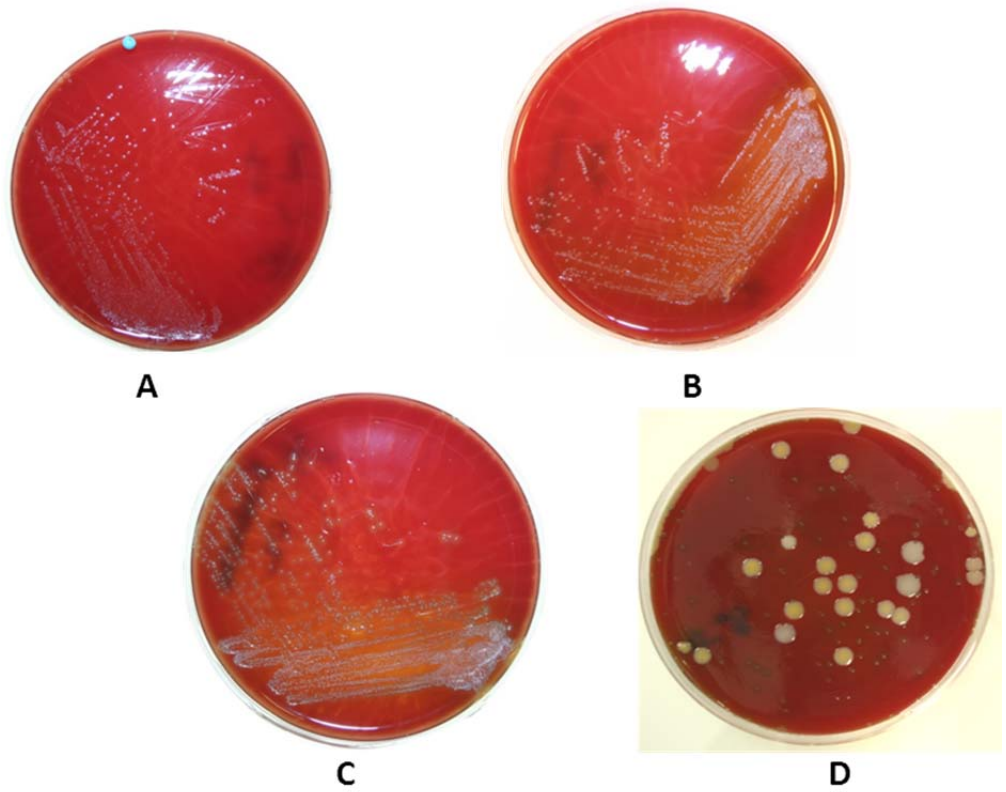

Figure 5.1 Isolates of *Gemella palacticanis* (A), *Lactobacillus animalis* (B) and *Streptococcus alactolyticus* (C) grown under aerobic conditions on blood agar showing the similarity of the colony morphology and challenges distinguishing them based on morphological differences especially on a mixed aerobic blood agar culture plate (D)

5.1.4. Determination of novel murine bacteria

It is clear from the non-cultural identification of the components of the mouse oral microbiota that there is diversity within specific micro-organisms, especially the *Streptococcus sp*. Using a 'best hit' approach interrogating the sequences against the BLAST database there appear to be variation within this genus. This analysis showed that there were 2 different species of *Streptococcus sp* that made up the majority of this genus; *Streptococcus alactolyticus*-like and *Streptococcus sanguinis*-like bacteria. In addition *Streptococcus intermedius*-like, *parasanguinis*-like, *equi*-like, *salivarius*-like and *peroris*-like bacteria were present in either single or double clones.

Whilst this approach allows intra-species determination of species the best hit approach has 2 major disadvantages. Firstly, based on degree of homology of the sequence (typically 96-99%) with the database over a given portion of the sequence (typically 96-99%) there are occasions when it is not possible to separate the identification based on ranking; a number of species of the micro-organism are identified with identical homology over identical proportions of the sequence. Ranking therefore becomes arbitrary. Secondly, despite setting a high threshold for identification of micro-organisms based on these criteria, sequences matches are not perfect i.e. 100% homology over 100% of sequence alignment. This leaves a degree of uncertainty regarding the identification which is compounded by the relatively short sequence lengths that are produced by this cloning and sequencing methodology (typically 900-1000bp).

It is possible that some of the micro-organisms detected are in reality novel murine species that have not yet been fully sequenced. This will only become apparent as the number and length of sequences produced increases or complete genome sequences are determined with further investigations. In addition, further biochemical and phenotypic analysis will help differentiate whether these are novel species. With this in mind all sequences are being stored on University computer network drives and consensus sequences being developed as representative of potentially novel murine strains of oral micro-organisms.

5.2. The effect of *Porphyromonas gingivalis* challenge on the oral commensal microbiota

5.2.1. Dysbiosis

Dysbiosis is defined as the unfavourable shift in the relative abundancies of individual components of the microbiota in disease when compared to health[178]. This is emerging as an important factor in the development of a number of diseases in other parts of the gastrointestinal tract (explored in more detail in section 2.9). Dysbiosis has been implicated in playing a role in antibiotic-associated diarrhoea[179, 180], inflammatory bowel disease[181-184], colorectal cancer[191-193] and obesity[194-197].

The data from the experiments where GF and SPF mice were challenged with *P. gingivalis* supports this idea of dysbiosis being an important driver of periodontal bone loss in this model. The effect of challenging an SPF mouse with *P. gingivalis* shows that there is an increase in total number of micro-organisms found in the microbiota when determined by cultural methods; a quantitative shift in the oral microbiome. In addition, culture techniques demonstrate a qualitative shift in the composition of the oral microbiome with respect to the major cultivable species following *P. gingivalis* challenge. These qualitative shifts are also detected when non-culture sequence methodology is used to assess the effect of *P. gingivalis* challenge. These quantitative and qualitative shifts in the oral commensal microbiota might be considered a dysbiosis as they are associated with increased alveolar bone loss (an unfavourable outcome).

5.2.2. Quantitative effects of *Porphyromonas gingivalis* superinfection

An important point to consider with these *P. gingivalis* challenge experiments is the nature of the inoculation process itself, specifically the amount of the infecting micro-organisms used. Data from our analysis of the commensal microbiota would indicate that the total number of bacteria within the oral cavity of an SPF mouse is in the order of 10^6 (see section 4.3 and 4.4). This is supported by real time PCR quantification of total bacteria in the same strain of mouse using the 16S rDNA gene in recent published data[198]. The protocol for oral gavage used in these studies requires 3 inoculations of 10^9 bacterial cells given on 3 occasions 48 hours apart. This is a 3 $\log_{10}$ increase following superinfection with *P. gingivalis* in the resident microbiota but interestingly is only very transient. The resultant effect is a sustained increase in bacterial load of approximately1-2 $\log_{10}$.

Studies that have looked at the mRNA expression in the gingival tissues following *P. gingivalis* inoculation have consistently shown significant increases in pro-inflammatory markers (TNF-α, IL-1β and IFN-α) in inoculated mice compared to controls[198]. These changes are transient and so would appear to be a manifestation of the inoculation with *P. gingivalis*. However, this challenge with *P. gingivalis* is sufficient to cause a lasting effect in shifting the amount and composition of the oral commensal microbiota to a new level (dysbiosis) which is then more damaging to the periodontal tissues. This is supported by the transfer of this oral commensal microbiota that has been modified by *P. gingivalis* challenge into a previously germ free conventionalised mouse which is then more destructive to the periodontal tissues. The effects on the inflammatory markers of this dysbiotic microbiota in the new host mouse would be interesting to compare to a challenged SPF mouse to examine if these elevations are induced by the dysbiotic microbiota.

Following this superinfection with *P. gingivalis* the resultant aftermath is a significant, sustained elevation in total microbial counts. In the aftermath it is worth remembering that *P. gingivalis* is only detectable at relatively low numbers (qualitative data supplied in section 4.4.4 and quantitative data based on qPCR [198]). Indeed, *P. gingivalis* is only a minor component of this dysbiotic microbiota representing only around one one hundred thousandth of the total bacteria present.

The oral gavage process itself is extremely inefficient and the majority of the *P. gingivalis* simply passes through the gastrointestinal tract and is excreted: even accepting coprophagia as a route for re-infection.

5.2.3. Variation between culture and non-culture techniques

There are again issues with differences in the compositional shifts detected by culture and non-culture techniques, specifically the changes in *Gemella sp*. In addition to the challenges with distinguishing the different micro-organisms based on morphological differences, and potential biases that may be introduced by suboptimal growth conditions favouring certain species over others, there is the added complexity of antibiotics being given as part of this challenge experimental protocol. The data from non-cultural analysis of the commensal microbiota suggests that the treating of SPF mice with antibiotics profoundly alters the composition of the microbiota especially the elimination of *Gemella sp*. It is only when these animals are challenged with *P. gingivalis* that *Gemella sp* is able to recolonise the oral environment.

5.2.4. The 'other' components of the commensal microbiota

The data presented in these studies show that there is a small but significant number of diverse micro-organisms that constitute the oral commensal microbiota of the mouse. These 'other' species typically represent less than 10% of the total oral microbiota but their importance is unclear from these data. As the library of sequences develops, this 'tail of diversity' in low number but detectable micro-organisms grows in number and variability.

This layer of complexity prevents more detailed definition of the mouse oral commensal microbiota. It may be that within these 'other' micro-organisms there are species that are able to act either directly to influence periodontal breakdown or through the commensal microbiota in a similar manner to *P. gingivalis*. In addition, the true diversity of the commensal microbiota cannot be fully determined; it may be a few tens or hundreds of species or it may approach the diversity of the human oral microbiome.

5.2.5. Specific, non-specific and ecological plaque hypotheses

If there turns out to be specific components in the 'other' micro-organisms that once identified can be shown to produce virulence factors and cause periodontal disease in animal models this would lend support to the specific plaque hypothesis (discussed in section 2.7.2).

It could be argued that the data presented in this thesis offers a way of unifying the 3 plaque hypotheses. Firstly, we have shown that the quantity of micro-organisms in the plaque biofilm is important in driving periodontal disease. Germ free mice do not develop bone loss, it is only when a commensal microbiota is present (SPF mice) that bone loss occurs. In addition, when this commensal microbiota is altered (quantitative increase) by *P. gingivalis* challenge the levels of bone loss increases. This supports the non-specific plaque hypothesis in that it is the amount of microbial load within the plaque biofilm that is the determining factor in the pathogenesis of periodontal disease. Secondly, the specific plaque hypothesis is supported by the data presented that challenge with a specific periodontal pathogen (*P. gingivalis*) induces periodontal bone loss. Finally, the dysbiosis induced by *P. gingivalis* challenge (quantitative and qualitative shift) is a change in the ecology of the subgingival microenvironment and total population structure leading to the appearance of normally minor constituents; it is then this that drives periodontal disease.

5.2.6. Concept of *Porphyromonas gingivalis* as a keystone pathogen

The concept of a keystone organism is prevalent in biology being first described by Robert Paine in the late 1960s in relation to the ecosystems of the ocean[427]. He coined the term 'keystone species' and defined it as an organism that is able to exhibit a disproportionally large influence on its environment relative to its low abundance. In this instance it was the effect a lower abundance starfish (*Pisaster ochraceus*) was having as a predator on the more multitudinous sea snail (*Tegula funebralis*). This concept has subsequently been explored in many diverse ecosystems[428].

Detection of *P. gingivalis* following inoculation into these SPF mice shows that it is only present as a small proportion of the total commensal microbiota. Despite this low level of colonisation the effects it is able to produce on the ecosystem has wide effects on the oral commensal microbiota. Based on these data it could be argued that *P. gingivalis* is acting as a keystone species[429]. The *P. gingivalis* gingipains cleave complement C5 to generate increased levels of C5a, this activates the C5a receptor that triggers inflammation[430]. In addition, the gingipains are also involved in subversive crosstalk with toll-like receptor 2 (TLR 2) through which there is impaired leukocyte killing. It is this orchestration of inflammatory disease by producing community wide effects whilst being present in only low numbers that qualifies it as a keystone pathogen. This is distinct from conventional infection models where the pathogenic organism uses its virulence determinants to induce inflammation to induce conditions that favour its growth. It is then able to outgrow the commensal microbiota, become the dominant species and induce disease e.g. *Salmonella typhimurim* induction of colitis (Figure 5.2).

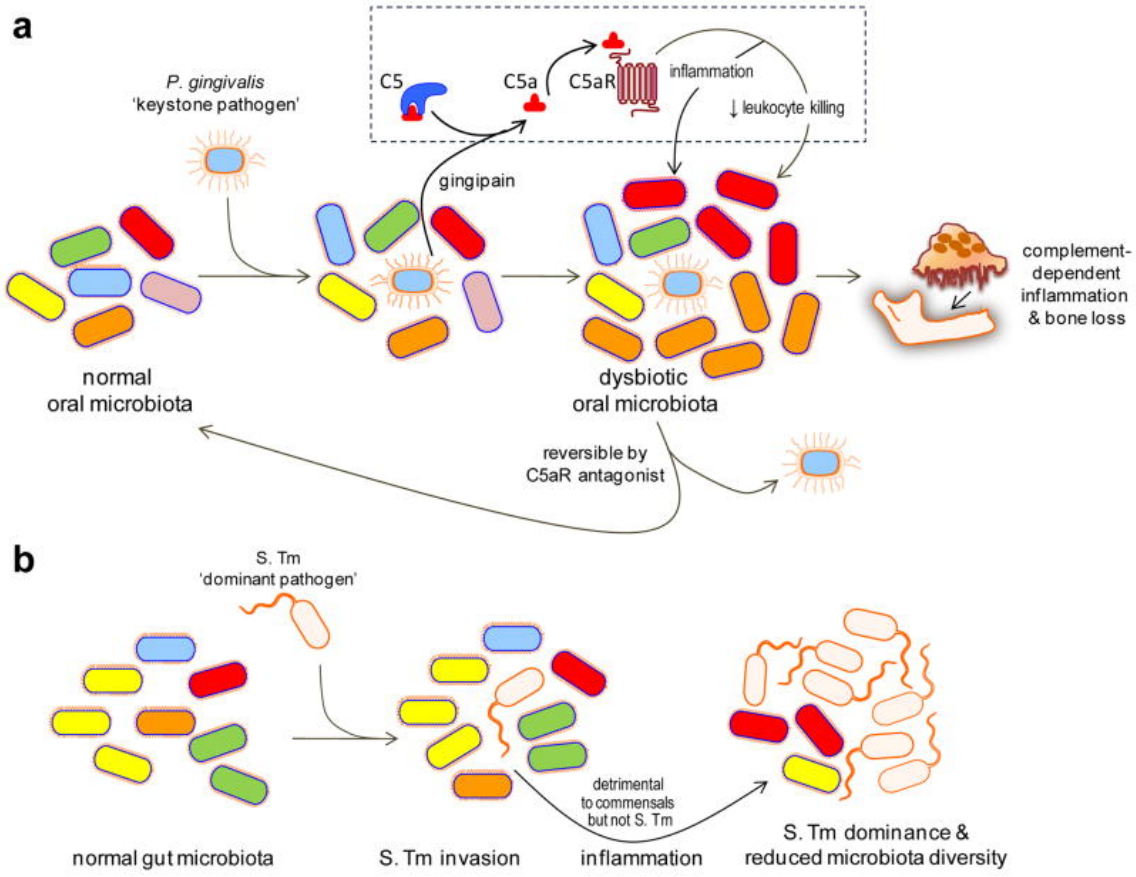

Figure 5.2 The action of a (a) keystone pathogen (*P. gingivalis*) by inducing community wide effects through a virulence factor (gingipains) interaction with host immune system (C5 and TLR2) despite being a low abundance constituent of the commensal microbiota leading to dysbiosis and disease. This contrasts with (b) the action of a conventional pathogen (*S. typhimurim*) which infects, causes inflammation which favours growth of the pathogen such that it becomes the dominant component of the commensal microbiota and causes disease. From Hajishengallis et al[430].

5.3. Influence of genetic background on the commensal microbiota

5.3.1. Importance of intact and functioning immune system

The periodontal tissues are a unique site in the human body in that they have highly specialised innate responses. The primary (and unique) tissue is the porous junctional epithelium that lies in direct proximity to the plaque biofilm on the surface of the tooth. This barrier is therefore under constant assault from the biofilm components and it is this that is responsible for one of the primary innate responses. The co-ordinated expression of intercellular adhesion molecules (ICAMs) and interleukin 8 (IL-8) enables the extravasation and migration into the junctional epithelium of neutrophils[71]. These form a transient and migratory population of innate cells that monitor and respond to the biofilm challenge to maintain the tissue homeostasis.

5.3.2. Effects of neutrophil deficiency

The data from the transmission experiments in the $CXCR2^{-/-}$ mice highlights the importance of a functioning immune system and especially the neutrophil. These mice are lacking the receptors for the mouse homologues of IL-8 on neutrophils. The tissue gradient of IL-8 is still present but the lack of receptors on the neutrophils means they are unable to sense this and migrate along the chemotactic gradient. This gradient is a vital first line of defence as it is chemotactic to neutrophils and allows a sentry population of cells to populate and monitor the periodontal tissues for microbial insult. In these mice the neutrophils are unable to migrate through the tissues and perform this function.

The result is spontaneous periodontal bone loss produced by an oral microbiota that might otherwise be considered not pathogenic. Indeed, when this microbiota is transmitted into a previously germ free mouse, by co-caging, it is unable to induce bone loss to the degree it could in the immune deficient mouse. Moreover, the data shows that following transfer into an immune-competent mouse the bone loss is less than a control mouse in that same strain (see section 4.8.2.3.).

In the knockout mouse with deficient neutrophil migration the composition of the microbiota is different from that in the control wild type, both in terms of total numbers of micro-organisms present and the constituent micro-organisms; dysbiotic. Despite this dysbiosis it is worth remembering that this oral microbiota does not have to be especially virulent or pathogenic as it is resident in an animal with limited immune responses. Consequently, when it is transferred into an animal with a functioning immune system it may not possess the virulence or pathogenicity that a microbiota that had developed in this host would have. Hence, levels of bone loss are less when compared to control mice with similar microbiota. This demonstrates the importance of the co-evolution of microbial biofilms with a host.

5.3.3. Neutrophil deficiency in human periodontal disease

The important role for the neutrophil in periodontal disease is highlighted by individuals with inherited neutrophil deficiencies who often exhibit severe periodontitis at young ages[431]. Leukocyte adhesion deficiency type 1 (LAD 1) is a genetic disorder characterised by loss of expression of integrin receptors on the surfaces of neutrophils that leads to impaired adhesion and chemotaxis. This condition is associated with periodontitis at very young ages[432-435]. In individuals with Chediak-Higashi syndrome there is impaired neutrophil function, number and chemotaxis and common association with severe periodontitis[436-438]. Other inherited conditions which are associated with impaired neutrophil function and early onset periodontal breakdown include Papillon-LeFevre syndrome[439-442]. These all go to highlight the pivotal role of the neutrophil in the pathogenesis of periodontal disease.

However, in del-1 knockout mice (Del1 is an endogenous inhibitor of neutrophil adhesion) there is an increased influx of neutrophils into the gingival tissues due to the absence of this inhibitor. These mice also exhibit increased levels of alveolar bone loss which is a manifestation of excessive accumulation of neutrophils in the periodontal tissues. This situation is akin to the excessive accumulation of neutrophils in the periodontal tissues in chronic periodontitis and results in periodontal bone loss.

5.4. Transmission of the commensal microbiota

In humans, the evidence supports a vertical mode of transmission of the oral commensal microbiota from mother (or carer) to child[443]. This is especially true for certain periodontal pathogenic micro-organisms such as *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia, Porphyromonas gingivalis* and *Prevotella nigrescens* when assessesed by PCR methodology[444] and N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis; an enzyme associated with. *T. forsythia*, *T. denticola* and *P. gingivalis*. The transmission from care-giver to child of *Aggregatibacter actinomycetemcomitans* has been estimated at 30-60% but the authors of this review cited the vertical transmission of *P. gingivalis* as rare[443]. However, in a later transmission study carried out by the same authors in a population in Java Indonesia they estimated the transmission rate of *P. gingivalis* at 46%[445].

5.4.1. Transmission of a normal oral commensal microbiota

The data presented in this thesis support a vertical mode of transmission of the oral commensal microbiota from mother to offspring. It is worth remembering that in these studies there are husbandry considerations that might bias the mode of transmission. Only male off spring of the same litter can be caged together, males from different litters cannot be mixed or violence and aggression results. In addition, males and females of any litter cannot be housed together as again fighting takes placing over breeding rites. This means that the only contact the male offspring has is with the mother during weaning. As a result their oral commensal microbiota can only be inherited by vertical transmission during this time frame. Any subsequent changes to

the commensal microbiota would therefore be brought about by horizontal transmission or by environmental sources.

That said the fact that this commensal oral microbiota is stable amongst male and female mice from different litters and at different ages would suggest that this mode of transmission does occur; especially as the microbiota of young mice is similar to very old mice kept under the same conditions. This would suggest the oral commensal microbiota is established early in life.

5.4.2. Transmission of a *P. gingivalis* influenced dysbiotic microbiota

The data presented in this thesis also supports horizontal transmsion of the commensal oral microbiota. This is shown by the co-caging experiments (sections 5.5-4.8) where the established commensal microbiota in a cohort of SPF mice is transmitted to germ free cage-mates in a horizontal fashion.

This is especially remarkable in the transmission of the *P. gingivalis* altered (dysbiotic) microbiota into the same strain of germ free mouse. It is all the more noteworthy that this dysbiotic microbiota is then able to induce increased alveolar bone loss in these previously germ free mice to a level similar to an SPF mouse challenged with a superinfection of *P. gingivalis*. The implication is that the dysbiosis is the key driving force in the periodontal inflammation in this model.

5.4.3. Transmission of a genetic background influenced dysbiotic microbiota

The horizontal transmission of the oral commensal microbiota from knockout mice into germ free mice is complicated by 2 factors. Firstly, there was not the availability of a germ free colony in the same background as the knockout mice (Balb/c). Therefore, transmission was into a different strain (C3H) with potential inherent inter-strain variability. Secondly, the oral commensal microbiota in the knockout strain is heavily influenced by the lack of an effective innate response mainly through lack of neutrophil migration. This means the microbiota is different (when compared to controls in the same background) and that this dysbiosis is a function of the genetics of the host i.e.

the genetic status of the host has a profound influence on the nature of the microbiome.

Despite these compromises it is interesting to note that the transmission of this microbiota into a previously germ free mouse (albeit in a different background strain) is only partial. There are differences in numbers of micro-organisms transferred and the proportions of the component bacteria. As a result this genetically selected dysbiotic microbiota is seemingly less pathogenic when transferred into an immune-competent host. Indeed, the resultant bone loss is less if compared to an SPF mouse in the same background as the previously germ free (C3H) mouse.

It is not clear from the studies presented here whether it is the genetically dysbiotic microbiota (a microbiota that is altered due to the host genetic status) that is in itself less virulent or whether it is the influence of the more readily functioning innate response. If the microbiota itself was less pathogenic and complete horizontal transfer was possible this would open up the possibilities of a pro-biotic approach to controlling periodontal inflammation in this mouse model.This line of investigation has led to the widespread use of pro-biotics in the field of gastro-enterology[446]. Probiotics have been shown to be more effective than placebo in the treatment of irritable bowel syndrome[447], effective in the management of antibiotic associated diarrhea[448] and have been investigated in the treatment of necrotising enterocolitis in pre-term infants[449].

This probiotic approach has been evaluated in the treatment of periodontal disease and the efficacy recently reviewed[450]. The delivery of the probiotic is the vital first step in permitting the micro-organism to colonise and influence its effects. This has been evaluated in different delivery systems such as *L. salivarius* [451] or *L. reuteri* [452]contained in a tablet which was shown to reduce the numbers of periopathogenic bacteria in plaque samples and administration of *L. reuteri* in a chewing gum which was shown to have beneficial short term effects on the reduction of pro-inflammatory cytokines in gingival crevicular fluid.

The use of milk for delivery of *L. casei* has also been investigated[453] where it was found to decrease the enzyme activity of MMPs and elastase although the clinical effects on bleeding index were not significant.

There was great heterogeneity between the studies relating to the efficacy of probiotics in the treatment but there was generally some form of clinical assessment of periodontal disease; probing pocket depth, bleeding index, clinical attachment level and plaque index, the majority of which showed some short term improvement following administration of the probiotic.

There has not been sufficient investigation as to the most efficacious bacteria to include in the probiotic. This is an area that could be investigated using the animal model used in the investigations reported in this thesis. This area is discussed further in relation to future work especially mono-infection experiments (section 6.2). The ideal probiotic agent would not be harmful to the host but able to colonise the oral cavity and become part of, and influence in a positive way, the commensal microbiome. It needs to be able to survive and replicate in this environment and so become established. Once established it needs to be able to exert an influence on the composition of the microbiota reversing any existing dysbiosis and shifting the microbiome from disease to health.

5.5. Conclusions

The investigations reported in this thesis offer one of the most extensive analyses and definition of the oral commensal microbiota of the mouse to date. We have defined the commensal microbiota by culture and non-culture techniques to a high degree and investigated alterations to this commensal microbiota due to a specific pathogen (*P. gingivalis*) and the influence of the genetic variations in the host and the effects on periodontal disease. We have also characterised the transmission patterns of this commensal microbiota between animals and the effects this has on the disease state in terms of a 'normal' microbiota and dysbiotic microbiota.

Chapter 6

Future research

6. Future research

The studies presented in this thesis open up some intriguing lines for further experiment and enquiry.

6.1. Significance of the 'other' components of the commensal microbiota

Non-cultural techniques have highlighted that despite a degree of stability within the mouse commensal microbiota there is a collection of low abundance micro-organisms that appear to be highly variable between individual mice. The full extent of these and their significance cannot be determined within the scope of this work.

One of the main conclusions of this work is the role of *P. gingivalis* in development of a dysbiotic commensal microbiota that drives the periodontal disease process in this mouse model. Given that the data supports the concept of *P. gingivalis* as a keystone species and that it is detectable in very low numbers following inoculations one could speculate that there may be significant components in the 'other' group of micro-organisms that may operate in a similar manner to *P. gingivalis*. However, one might expect to see accelerated bone loss if this was the case unless they require a different shift in the commensal microbiota to reach a threshold level at which they can exert their influence.

Estimates put the levels of *P. gingivalis* at one hundred-thousandth of the total oral commensal microbiota. At this level using the conventional sequencing techniques one might have to clone and sequence 100,000 transformants before *P. gingivalis* or an equivalent low abundance member of the 'other' group before they were detected. Clearly this is not a feasible option.

It would therefore be useful to use the next generation sequencing technologies that are available as these can produce many tens or hundreds of thousands of sequences per sample. This would provide a twofold advantage over the current technique. Firstly, with the quantity of sequences produced there would be generation of greater detail in this 'other' group and any significant differences would be highlighted.

Secondly, construction of such huge libraries would enable finer determination of the sequences and resolution to a species level that might confirm the presence of novel mouse micro-organisms. At present homology of sequences generated with BLAST is >96% but rarely 100%. With more sequences one would be able to generate greater accuracy in the consensus sequences.

The amount of DNA that can be extracted from a swab from a single mouse is a limiting factor for these analyses. Further optimisation of sample collection, processing and storage would need to take place before this technology could be applied. In addition, there are issues with errors in reading the sequences that are inherent in these systems along with considerable computing storage space and bioinformatic analysis resources.

6.2. Importance of specific organisms within the commensal microbiota to periodontal bone loss

In these studies we have investigated the importance of the normal and the dysbiotic commensal microbiota in periodontal disease. We have shown an intact commensal microbiota is required for alveolar bone loss. However, it is unclear exactly which of the component bacteria may be important in periodontal inflammation.

To address this, a series of mono-infection oral gavage experiments could be carried out in specific pathogen free (SPF) mice. In these experiments, isolates of specific components of the commensal microbiota could be inoculated into the SPF mice in the same manner as *P. gingivalis*. Initially, the major components of the mouse oral commensal microbiota (*Gemella sp*, *Lactobacillus sp* and *Streptococcus sp*) could be isolated. In addition, potential pathogenic micro-organims in the 'other' group (such as *Bacteroides sp*, *Acinetobacter sp* and *Staphylococcus sp*) could be isolated. Isolation of these micro-organisms would require selective media to favour their growth over the other commensal micro-organisms present. Once growth was established confirmation of the correct species of micro-organism could be carried out by molecular means and possibly sequence of the whole genome.

These micro-organisms could then be inoculated into the SPF mice and periodic monitoring of the developing commensal microbiota could be made by culture and non-culture techniques for evidence of dysbiosis. Following sacrifice, alveolar bone loss could be assessed and compared to control SPF mice.

The most likely candidate micro-organisms for investigation in this way would be the mouse derived *Gemella sp*, *Streptococcus sp*, *Lactobacilus sp* and *Staphylococcus sp*. In addition, to mono-infection inocula could be prepared of various combinations of these candidate species. One might also investigate immune response to the infecting micro-organism through bacteria-specific IgG levels assessed by ELISA, the expression of various inflammatory markers in the gingival tissues through qPCR to quantify mRNA levels of IL1, IL8, TNFα or use immuno-histochemistry to investigate inflammatory cell types in these tissues.

This type of approach has been pioneered recently in respect to the nucleotide-binding oligomerization domain-containing protein 1 (Nod-1)[382]. These investigators used Nod-1 (a cytoplasmic pattern recognition for bacterial peptidoglycan) knockout mice and showed that this receptor is required for alveolar bone loss following ligature induced inflammation. However, they also linked this with the observation that there was significant accumulation of a specific *Aggregatibacter actinomycetemcomitans*-like bacteria (termed NI1060) at the site of the ligature. Significantly, mono-colonisation with this bacteria (NI1060) was sufficient to induce alveolar bone loss in germ free mice in a Nod-1 dependent manner. This is unlike our findings where mono-inoculation with *P. gingivalis* failed to induce alveolar bone loss in germ free mice. These data are significant in that they offer a potential mechanism in which a single component of the oral commensal microbiota might induce periodontal bone loss through pattern recognition receptor (Nod-1) and the ensuing inflammatory cascade (Figure 6.1). If this bacteria (NI1060) is a mouse strain of *A. actinomycetemcomitans* its ability to induce bone loss through this pathway is a useful model of periodontal disease to explore host/bacteria interactions.

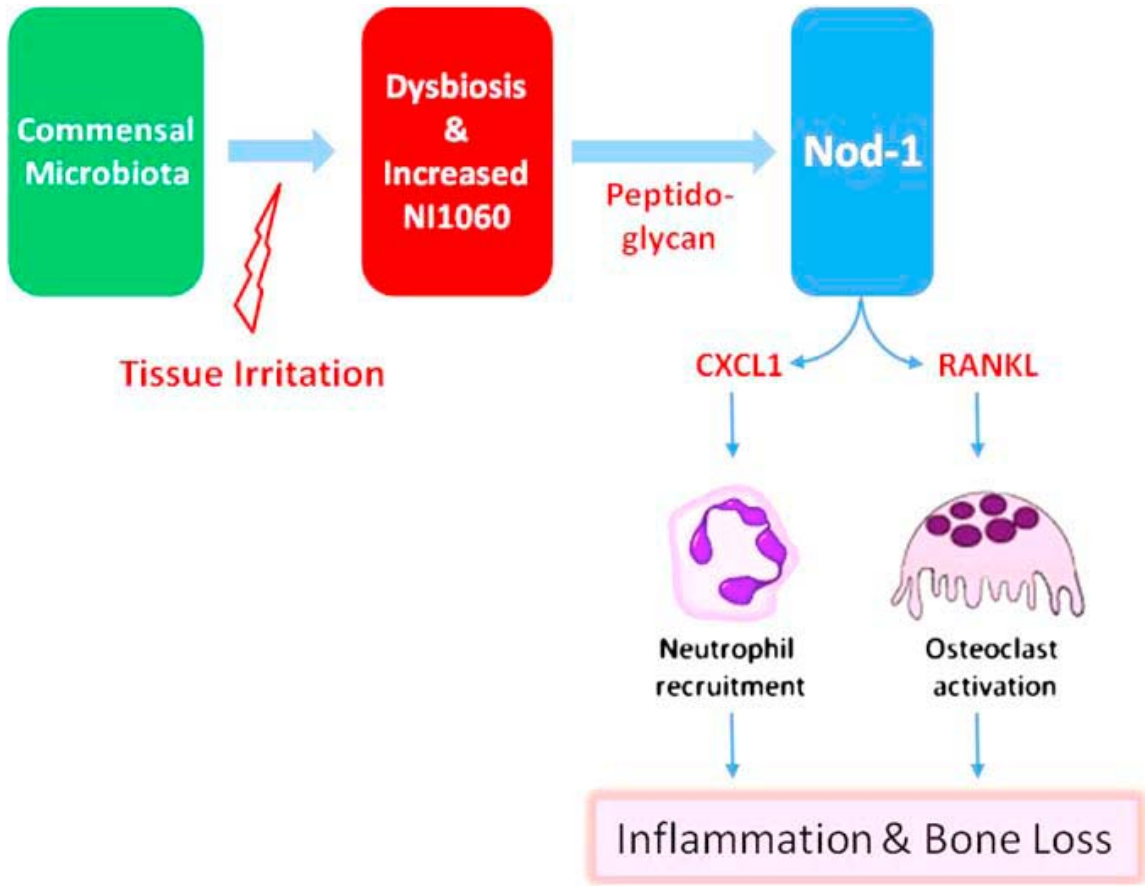

Figure 6.1 The normal commensal microbiota is disrupted by the placement of a ligature with tissue irritation and dysbiosis in which an increase in a specific *A. actinomycetemcomitans*-like bacteria (NI1060) induces elevated levels of bacterial peptidoglycan and activation of Nod-1. This leads to increased neutrophil recruitment through CXCL1 and increased osteoclast activation through RANKL and resultant inflammatory alveolar bone loss. Figure from Cell, Host & Microbe[454].

6.3. Further investigation of *Porphyromonas gingivalis* as a keystone species

The concept of *P. gingivalis* as a keystone species in the pathogenesis of periodontal disease is a relatively new idea. For this idea to gain further validity the mechanisms through which it can influence such a large effect despite being present in low numbers needs to be elucidated. It might be that with re-interpretation of the current literature the evidence will grow (e.g. gingipains, LPS, CPS, fimbriae). Equally new areas of research such as membrane vesicles might offer an insight into the mode of action of *P. gingivalis* as a keystone species.

Probably the most significant issue to resolve in this mouse model is the dosage of *P. gingivalis* that is used. The levels of bacteria that are introduced by inoculation are not physiological levels and constitute a superinfection. The ethos behind the keystone hypothesis is that the organism is present in low numbers. Following oral gavage with *P. gingivalis* the levels of this micro-organism are low. However, it is not clear if more physiological levels of *P. gingivalis* can induce similar levels of dysbiosis and periodontal inflammation. An inoculation experiment whereby decreasing doses of *P. gingivalis* are given would be required to elucidate this unknown. It might be that there is a minimum threshold level of *P. gingivalis* that is required to induce dysbiosis. This could be significant as it might offer an explanation as to why *P. gingivalis* (or other components of the oral commensal microbiota) can be present without inducing alveolar bone if they are below the threshold levels required to induce dysbiosis. How these levels compared with physiological levels would then provide insight into the role of *P. gingivalis* in this model and is more akin to human periodontal inflammation.

6.4. Transmission of the dysbiotic commensal microbiota

One of the more surprising findings presented in this thesis is the fact that the *P. gingivalis* induced dysbiotic microbiota was transmitted into a germ free mouse and that then led to alveolar bone loss to the same degree as a *P. gingivalis* challenged SPF mouse.

Whilst statistically this experiment had sufficient power it would be beneficial to repeat with increased numbers in each group. Although the numbers of mice included in these studies satisfy power calculations at 80% with a significance of $p<0.05$ the nature of the bone loss data and the microbiological data is such that there was a large standard deviation. Larger numbers might be expected to narrow this deviation and highlight any changes that might be due to experimental intervention.

This would give the additional benefit of allowing further investigation of whether the innate/inflammatory response in the animals which receive a Pg "altered microbiota" is the same as in a conventionally challenged SPF mouse. Cell types could be examined through immunohistochemistry (IHC) and expression of pro-inflammatory cytokines through qPCR.

The major issue with this methodology is the need for all female and appropriately aged mice within the GF and SPF colonies. Routinely only 2 breeding pairs are available in each colony so co-ordination to achieve sufficient mouse numbers is very difficult meaning that for each individual experiment numbers may be limited. Hence, the need for repeat experiments to offer supporting data.

6.5. Adaptation of the oral gavage model of experimental periodontitis

The oral gavage model of experimental periodontits has served research in this area well for many years. However, the data from these studies brings into question the use of antibiotics. The original pioneering studies required antibiotics to suppress the commensal oral microbiota with a view to allowing the inoculated micro-organism to gain a foothold and colonise. The data presented in this thesis has shown that it is the commensal microbiota that is a key determinant in driving periodontal inflammation so suppression of this with antibiotics would appear to be contraindicated. In addition, we have shown that levels of periodontal bone loss are similar in SPF mice challenged with *P. gingivalis* whether antibiotics are administered or not. Moreover, *P. gingivalis* is detectable from inoculated mice that have not been pre-treated with antibiotics. The validity of this would need to be further investigated in other mice strains.

6.6. Conclusions

One of the key issues highlighted in this thesis is the transition of a stable oral commensal microbiota which is in harmony with the host and does not result in damage to an equally stable dysbiotic microbiota that is pathogenic to the host and results in breakdown of the periodontal tissues. Whilst our data supports the idea that

the dysbiotic microbiota is stable over the experimental period (6 weeks) this is relatively short term and the long term stability needs to be established. Is this dysbiotic shift more permanent and the long term driver of chronic periodontal inflammation?

This could be established *in vitro* using a chemostat to develop a commensal microbiota akin to that of the SPF mice and then to induce dysbiosis with *P. gingivalis* (or other micro-organism). The stability of this dysbiotic commensal microbiota could then be assessed as could ways of converting this dysbiosis back to a more normal commensal microbiota with use of therapeutic agents or using probiotic bacteria. This could also be explored in the mouse model used in this thesis.

Relating these findings to the situation in human periodontal disease might offer novel therapeutic interventions especially in relation to probiotics. Although the human oral microbiome is considerably more varied and complex, these principles might still be useful. The use of high throughput 'bench top' next generation sequencing is already allowing rapid and cost effective analysis of the human oral microbiome. This has opened the door for applying these techniques longitudinally in which one might examine global changes in the oral microbiome to establish which components might be damaging and which might be protective. Modification of a dysbiotic microbiota might then be possible through re-introduction of the protective elements.

We are on the cusp of being able to routinely generate specific individual-based analyses of the oral microbiome for our periodontal patients and that might offer us novel insights into how to modify their dysbiotic microbiome and convert it back to a 'healthy' microbiome. Exciting times ahead!

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Appendices

1. Mouse data sheets

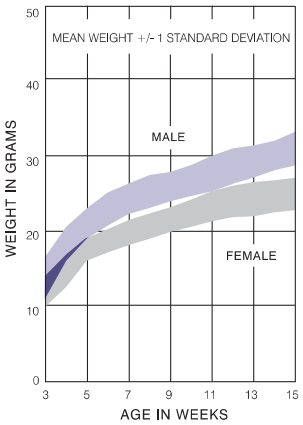
1.1. C3H/HeNCrl mouse

C3H Mice

STRAIN CODE: 025

Weight in Grams	MALE Approximate Age in Days	MALE Price	FEMALE Approximate Age in Days	FEMALE Price
Up to 12	Up to 23	19.65	Up to 25	21.10
13–14	24–26	22.60	26–28	24.10
15–16	27–28	23.10	29–34	24.95
17–18	29–31	24.10	35–42	25.80
19–21	32–42	25.80	43–56	27.75
22–24	43–58	29.15	57–75	31.00
25 plus	Prices upon request		Prices upon request	
Retired breeders		18.55		18.55
Littermates 21 days old only		28.90		28.90
Lactating mouse with litter				174.25
Timed/untimed pregnant				149.15

NOMENCLATURE C3H/HeNCrl **ORIGIN** From a cross of a Bagg albino female and a DBA male by Strong in 1920. A litter of 4 females and 2 males sent to Andervont in 1930, then to Heston at F35. To NIH in 1951 from Heston at F57. To Charles River in 1974 from NIH. **COAT COLOR** Agouti (wild-type). **RESEARCH APPLICATION** safety and efficacy testing, oncology, neurological disorders, retinal degeneration **MHC HAPLOTYPE** $H2^k$



Information from Charles River International at http://www.criver.com/en-US/ProdServ/ByType/ResModOver/ResMod/Pages/BALBcMouse.aspx

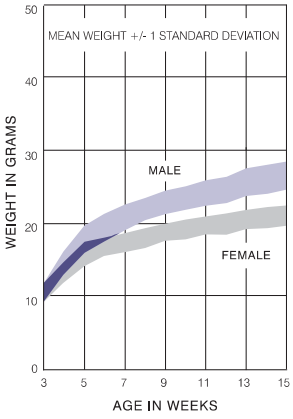
1.2. Balb/c AnNCrl

BALB/c Mice

STRAIN CODE: 028

Weight in Grams	MALE Approximate Age in Days	MALE Price	FEMALE Approximate Age in Days	FEMALE Price
Up to 12	Up to 22	18.55	Up to 25	19.60
13–14	23–26	21.50	26–28	22.75
15–16	27–31	22.25	29–35	23.55
17–18	32–38	22.90	36–53	24.25
19–21	39–49	24.95	54–77	26.40
22–24	50–70	28.20	78–98	29.60
25 plus	Prices upon request		Prices upon request	
Retired breeders		17.80		17.80
Littermates 21 days old only		27.60		27.60
Lactating mouse with litter				163.20
Timed/untimed pregnant				138.60

NOMENCLATURE BALB/cAnNCrl **ORIGIN** H.J. Bagg developed the "Bagg albino" in 1913 from stock from an Ohio pet dealer. Inbred in 1923 by McDowell. To Snell in 1932 at F26, then to Andervont in 1935. To NIH in 1951 from Andervont at F72. To Charles River in 1974 from NIH. **COAT COLOR** White (Albino).
RESEARCH APPLICATION general multipurpose model, hybridoma development, monoclonal antibody production, infectious disease **MHC HAPLOTYPE** $H2^d$



Information from Charles River International at http://www.criver.com/EN-US/PRODSERV/BYTYPE/RESMODOVER/RESMOD/Pages/C3HMouse.aspx

1.3. CXCR2 -/-/J

Information from The Jackson Laboratory International at
http://jaxmice.jax.org/strain/002724.html

Strain Information

Former Names	C.129S2(B6)-*Il8rb*^{tm1Mwm}/J (Changed: 30-NOV-09)
	Cmkar2^{tm} (Changed: 15-DEC-04)
	IL8R (Changed: 15-DEC-04)
Type	Congenic; Mutant Strain; Targeted Mutation;
	Additional information on Genetically Engineered and Mutant Mice.
	Visit our online *Nomenclature tutorial*.
	Additional information on Congenic nomenclature.
Mating System	Homozygote x Heterozygote (Female x Male) 26-FEB-08
Species	laboratory mouse
Background Strain	BALB/c
Donor Strain	B6;129S-*Il8rb*^{tm1Mwm} (129S2 derived D3 ES cell line)
Generation	N8F15N1F13 (27-NOV-11) Generation Definitions
Donating Investigator	Dr. Mark W. Moore, Deltagen

Appearance
albino
Related Genotype: *A/A Tyrp1*^{b}*/Tyrp1*^{b} *Tyr*^{c}*/Tyr*^{c}

Description

Mice homozygous for the this targeted mutation are viable and fertile although the reproductive rate is lower than normal wildtype siblings. Homozygous mutant mice have splenomegaly, lymphadenopathy, and impaired neutrophil migration. Formerly referred to as *Cmkar2*, chemokine (C-X-C) receptor 2; also called Il8r, interleukin 8 receptor.

Control Information

Control
000651 BALB/cJ

Considerations for Choosing Controls

2. John's Transport Media

Yeast Extract	0.5g $100ml^{-1}$
Protease Peptone	0.1g $100ml^{-1}$
Sodium Chloride	0.85g $100ml^{-1}$
Cysteine Hydrochloride	0.05g $100ml^{-1}$
Sodium Hydrogen Phosphate	0.085g $100ml^{-1}$
Tween 80	0.1ml $100ml^{-1}$
Glycerol	15ml $100ml^{-1}$
De-ionised water	85ml $100ml^{-1}$

Adjust to pH7.0 +/- 0.1 with 1M Sodium Hydroxide

Autoclave and decant into sterile Universal Pots

Store at $4^{o}C$

3. MALDI-TOF Results

Bruker Daltonics MALDI Biotyper Classification Results

Project Info

Project Name:	**2010.07.26**
Project Description:	MARK001
Project Owner:	microbiologyrlh@FLEX-PC
Project Creation Date/Time:	2010-07-26 18:16:28.906
Project Analyte Count:	24
Project Type:	RUO (Research Use Only)

Result Overview

AnalyteName	AnalyteID	Organism(best match)	ScoreValue	Organism(second best match)	ScoreValue
A1 (+++)		Staphylococcus capitis	2.315	Staphylococcus capitis	2.121
A2 (+)		Staphylococcus epidermidis	1.766	Staphylococcus epidermidis	1.716
A3 (-)		**not reliable identification**	1.6	**not reliable identification**	1.572
A4 (++)		Staphylococcus hominis	2.016	Staphylococcus hominis	1.932
A5 (++)		Staphylococcus capitis	2.251	Staphylococcus capitis	2.135

A6 (+)		Enterococcus faecalis	1.803	Enterococcus faecalis	1.787
A7 (-)		**not reliable identification**	1.445	**not reliable identification**	1.405
A8 (+)		Staphylococcus lentus	1.902	**not reliable identification**	1.468
A9 (-)		**not reliable identification**	1.367	**not reliable identification**	1.346
A10 (++)		Staphylococcus lentus	2.001	**not reliable identification**	1.592
A11 (++)		Staphylococcus aureus	2.192	Staphylococcus aureus	2.118
A12 (-)		**not reliable identification**	1.463	**not reliable identification**	1.339
B1 (-)		**not reliable identification**	1.345	**not reliable identification**	1.334
B2 (-)		**not reliable identification**	1.349	**not reliable identification**	1.296
B3 (++)		Enterobacter asburiae	2.064	Enterobacter kobei	2.029
B4 (++)		Enterobacter asburiae	2.268	Enterobacter kobei	2.241
B5 (-)		**not reliable identification**	1.327	**not reliable identification**	1.324

B6 (++)		Staphylococcus aureus	2.251	Staphylococcus aureus	2.192
B7 (++)		Enterobacter asburiae	2.255	Enterobacter kobei	2.192
B8 (++)		Staphylococcus aureus	2.215	Staphylococcus aureus	2.177
B9 (++)		Staphylococcus aureus	2.189	Staphylococcus aureus	2.082
B10 (++)		Staphylococcus aureus	2.185	Staphylococcus aureus	2.154
B11 (-)		**no peaks found**	< 0	**no peaks found**	< 0
B12 (++)		Escherichia coli	2.281	Escherichia coli	2.14

Meaning of Score Values

Range	Description	Symbols	Color
2.300 ... 3.000	**highly probable species identification**	**(+++)**	green
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**	green
1.700 ... 1.999	**probable genus identification**	**(+)**	yellow
0.000 ... 1.699	**not reliable identification**	**(-)**	red

Analyte1

Analyte Name: A1

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(+++)	Staphylococcus capitis ssp urealyticus DSM 6717T DSM	2.315	74703
2(++)	Staphylococcus capitis Mb18717_1 CHB	2.121	29388
3(-)	Staphylococcus capitis ssp capitis DSM 6180 DSM	1.663	72758
4(-)	Staphylococcus pasteuri DSM 10656T DSM	1.475	45972
5(-)	Staphylococcus lugdunensis DSM 4804T DSM	1.473	28035
6(-)	Staphylococcus lugdunensis DSM 4806 DSM	1.455	28035
7(-)	Staphylococcus lugdunensis DSM 4805 DSM	1.454	28035

Analyte2

Analyte Name: A2

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(+)	Staphylococcus epidermidis ATCC 14990T THL	1.766	1282
2(+)	Staphylococcus epidermidis 10547 CHB	1.716	1282
3(-)	Staphylococcus epidermidis CCM 4505 CCM	1.679	1282
4(-)	Staphylococcus epidermidis ATCC 12228 THL	1.572	1282
5(-)	Staphylococcus pasteuri DSM 10656T DSM	1.528	45972
6(-)	Staphylococcus warneri CCM 2604 CCM	1.472	1292
7(-)	Staphylococcus capitis Mb18717_1 CHB	1.388	29388
8(-)	Staphylococcus pasteuri DSM 10657 DSM	1.345	45972
9(-)	Staphylococcus warneri DSM 20036 DSM	1.318	1292

Analyte3

Analyte Name: A3

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Staphylococcus sciuri ssp sciuri DSM 20345T DSM	1.6	147467
2(-)	Staphylococcus sciuri ssp rodentium DSM 16827T DSM	1.572	147469
3(-)	Staphylococcus sciuri ssp sciuri DSM 6671 DSM	1.542	147467
4(-)	Staphylococcus sciuri ssp carnaticus DSM 15613T DSM	1.539	147468
5(-)	Staphylococcus vitulinus DSM 15615T DSM	1.5	71237
6(-)	Staphylococcus fleurettii DSM 13212T DSM	1.369	150056
7(-)	Bacteroides fragilis ATCC 25285T PNU	1.352	817
8(-)	Bacteroides fragilis MB_9009_05 THL	1.276	817

Analyte4

Analyte Name: A4

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus hominis ssp novobiosepticus DSM 15614T DSM	2.016	145393
2(+)	Staphylococcus hominis Mb18788_1 CHB	1.932	1290
3(+)	Staphylococcus hominis 18 ESL	1.831	1290
4(-)	Staphylococcus hominis ssp hominis DSM 20330 DSM	1.58	145391
5(-)	Staphylococcus hominis ssp hominis DSM 20328T DSM	1.489	145391
6(-)	Staphylococcus hominis ssp hominis DSM 20329 DSM	1.391	145391
7(-)	Staphylococcus haemolyticus 10024 CHB	1.302	1283
8(-)	Lactobacillus plantarum DSM 20205 DSM	1.027	1590
9(-)	Burkholderia phenazinium DSM 10684T HAM	1.019	60549

Analyte5

Analyte Name: A5

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.859

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus capitis ssp urealyticus DSM 6717T DSM	2.251	74703
2(++)	Staphylococcus capitis ssp capitis DSM 20326T DSM	2.135	72758
3(++)	Staphylococcus capitis ssp capitis DSM 6180 DSM	2.124	72758
4(++)	Staphylococcus capitis ssp capitis DSM 20325 DSM	2.109	72758
5(+)	Staphylococcus capitis ssp capitis DSM 20327 DSM	1.804	72758
6(-)	Staphylococcus capitis Mb18717_1 CHB	1.694	29388
7(-)	Staphylococcus lugdunensis DSM 4806 DSM	1.543	28035
8(-)	Staphylococcus lugdunensis DSM 4804T	1.526	28035

Analyte6

Analyte Name: A6

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(+)	Enterococcus faecalis ATCC 7080 THL	1.803	1351
2(+)	Enterococcus faecalis 20247_4 CHB	1.787	1351
3(+)	Enterococcus faecalis DSM 20409 DSM	1.732	1351
4(-)	Enterococcus faecalis DSM 20478T DSM	1.612	1351
5(-)	Enterococcus faecalis DSM 6134 DSM	1.611	1351
6(-)	Enterococcus faecalis DSM 2570 DSM	1.592	1351
7(-)	Enterococcus faecalis ATCC 29212 CHB	1.585	1351
8(-)	Enterococcus faecalis DSM 20371 DSM	1.567	1351
9(-)	Clostridium sp[1] 15_759 IBS	1.091	1485
10(-)	Microbacterium liquefaciens HKI 11374 HKJ	1.085	33918

Analyte7

Analyte Name: A7

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Streptococcus infantis DSM 12492T DSM	1.445	68892
2(-)	Streptococcus australis DSM 15627T DSM	1.405	113107
3(-)	Streptococcus pneumoniae DSM 11865 DSM	1.382	1313
4(-)	Lactobacillus alimentarius DSM 20181 DSM	1.377	1602
5(-)	Lactobacillus sharpeae DSM 20504 DSM	1.363	1626
6(-)	Streptococcus pneumoniae besSt29 THL	1.3	1313
7(-)	Lactobacillus sharpeae DSM 20506 DSM	1.281	1626
8(-)	Enterococcus faecium 20218_1 CHB	1.278	1352
9(-)	Enterococcus faecalis ATCC 7080 THL	1.269	1351
10(-)	Streptococcus pseudopneumoniae DSM 18670T DSM	1.266	257758

Analyte8

Analyte Name: A8

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(+)	Staphylococcus lentus DSM 6672 DSM	1.902	42858
2(-)	Staphylococcus sciuri ssp carnaticus DSM 15613T DSM	1.468	147468
3(-)	Staphylococcus vitulinus DSM 15615T DSM	1.435	71237
4(-)	Staphylococcus vitulinus DSM 9930 DSM	1.421	71237
5(-)	Staphylococcus sciuri ssp sciuri DSM 20345T DSM	1.415	147467
6(-)	Staphylococcus lentus DSM 20352T DSM	1.409	42858
7(-)	Staphylococcus vitulinus DSM 9931 DSM	1.389	71237
8(-)	Staphylococcus sciuri ssp sciuri DSM 6671 DSM	1.33	147467

Analyte9

Analyte Name: A9

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Streptococcus massiliensis DSM 18628T DSM	1.367	313439
2(-)	Lactobacillus sharpeae DSM 20504 DSM	1.346	1626
3(-)	Streptococcus sp DSM 20398 DSM	1.329	1301
4(-)	Staphylococcus hominis ssp novobiosepticus DSM 15614T DSM	1.306	145393
5(-)	Lactobacillus amylophilus DSM 20533T DSM	1.303	1603
6(-)	Streptococcus gordonii DSM 20568 DSM	1.3	1302
7(-)	Lactobacillus sharpeae DSM 20506 DSM	1.295	1626
8(-)	Streptococcus salivarius DSM 20560T DSM	1.253	1304
9(-)	Staphylococcus muscae DSM 7068T DSM	1.25	1294

Analyte10

Analyte Name: A10

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.859

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus lentus DSM 6672 DSM	2.001	42858
2(-)	Staphylococcus lentus DSM 20352T DSM	1.592	42858
3(-)	Staphylococcus vitulinus DSM 9930 DSM	1.549	71237
4(-)	Staphylococcus vitulinus DSM 9931 DSM	1.529	71237
5(-)	Staphylococcus sciuri ssp sciuri DSM 20345T DSM	1.451	147467
6(-)	Staphylococcus sciuri ssp carnaticus DSM 15613T DSM	1.384	147468
7(-)	Staphylococcus sciuri ssp rodentium DSM 16827T DSM	1.368	147469
8(-)	Staphylococcus vitulinus DSM 15615T DSM	1.343	71237
9(-)	Staphylococcus fleurettii DSM 13212T DSM	1.224	150056

Analyte11

Analyte Name: A11

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus aureus ATCC 33591 THL	2.192	1280
2(++)	Staphylococcus aureus ATCC 29213 THL	2.118	1280
3(++)	Staphylococcus aureus ssp aureus DSM 4910 DSM	2.115	46170
4(++)	Staphylococcus aureus ssp aureus DSM 346 DSM	2.042	46170
5(++)	Staphylococcus aureus ATCC 25923 THL	2.034	1280
6(+)	Staphylococcus aureus ssp aureus DSM 3463 DSM	1.991	46170
7(+)	Staphylococcus aureus ssp aureus DSM 20232 DSM	1.991	46170
8(+)	Staphylococcus aureus ATCC 33862 THL	1.963	1280
9(+)	Staphylococcus aureus ssp aureus DSM 20491 DSM	1.936	46170

Analyte12

Analyte Name: A12

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.859

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Streptococcus pneumoniae besSt29 THL	1.463	1313
2(-)	Streptococcus infantis DSM 12492T DSM	1.339	68892
3(-)	Streptococcus sanguinis CIP 55_128T CIP	1.334	1305
4(-)	Streptococcus canis DSM 20715T DSM	1.312	1329
5(-)	Streptococcus pneumoniae ATCC 49619 THL	1.262	1313
6(-)	Streptococcus hyointestinalis DSM 20770T DSM	1.253	1337
7(-)	Streptococcus pyogenes DSM 11728 DSM	1.244	1314
8(-)	Rhizobium radiobacter DSM 30147T HAM	1.212	311403
9(-)	Streptococcus sanguinis DSM 20567T DSM	1.204	1305
10(-)	Streptococcus ovis DSM 16829T DSM	1.203	82806

Analyte13

Analyte Name: B1

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Lactobacillus sharpeae DSM 20504 DSM	1.345	1626
2(-)	Streptococcus pneumoniae besSt29 THL	1.334	1313
3(-)	Lactobacillus sharpeae DSM 20506 DSM	1.313	1626
4(-)	Lactobacillus sharpeae DSM 20505T DSM	1.297	1626
5(-)	Corynebacterium urealyticum DSM 7110 DSM	1.275	43771
6(-)	Streptococcus suis DSM 9684 DSM	1.258	1307
7(-)	Streptococcus pneumoniae DSM 11868 DSM	1.247	1313
8(-)	Streptococcus salivarius DSM 20560T DSM	1.242	1304

Analyte14

Analyte Name: B2

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Enterococcus faecalis ATCC 7080 THL	1.349	1351
2(-)	Streptococcus downei DSM 5635T DSM	1.296	1317
3(-)	Streptococcus hyovaginalis DSM 12219T DSM	1.264	149015
4(-)	Enterococcus faecalis ATCC 29212 CHB	1.264	1351
5(-)	Streptococcus infantis DSM 12492T DSM	1.251	68892
6(-)	Lactobacillus sharpeae DSM 20504 DSM	1.246	1626
7(-)	Streptococcus constellatus ssp pharyngis DSM 17475T DSM	1.241	184250
8(-)	Pseudomonas citronellolis DSM 50332T HAM	1.237	53408
9(-)	Streptococcus pneumoniae DSM 11866 DSM	1.234	1313

Analyte15

Analyte Name: B3

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.859

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Enterobacter asburiae DSM 17506T DSM	2.064	61645
2(++)	Enterobacter kobei DSM 13645T DSM	2.029	208224
3(+)	Enterobacter cloacae MB11506_1 CHB	1.973	550
4(+)	Enterobacter ludwigii DSM 16688T DSM	1.948	299767
5(+)	Enterobacter asburiae CCM 4032 CCM	1.879	61645
6(+)	Enterobacter cancerogenus DSM 17580T DSM	1.871	69218
7(+)	Enterobacter cloacae MB_5277_05 THL	1.842	550
8(+)	Enterobacter cloacae MB_8779_05 THL	1.827	550
9(+)	Enterobacter hormaechei ssp hormaechei DSM 12409T DSM	1.819	301105

Analyte16

Analyte Name: B4

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Enterobacter asburiae DSM 17506T DSM	2.268	61645
2(++)	Enterobacter kobei DSM 13645T DSM	2.241	208224
3(++)	Enterobacter ludwigii DSM 16688T DSM	2.123	299767
4(++)	Enterobacter cloacae MB11506_1 CHB	2.112	550
5(++)	Enterobacter cloacae DSM 46348 DSM	2.071	550
6(++)	Enterobacter asburiae CCM 4032 CCM	2.07	61645
7(++)	Enterobacter cloacae ssp dissolvens DSM 16657T HAM	2.05	69219
8(++)	Enterobacter cloacae DSM 30062 DSM	2.017	550
9(++)	Enterobacter cloacae DSM 6234 DSM	2.017	550
10(++)	Enterobacter cloacae 13159_1 CHB	2.01	550

Analyte17

Analyte Name: B5

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	Streptococcus pneumoniae besSt29 THL	1.327	1313
2(-)	Lactobacillus sharpeae DSM 20506 DSM	1.324	1626
3(-)	Lactobacillus sharpeae DSM 20505T DSM	1.28	1626
4(-)	Lactobacillus sharpeae DSM 20504 DSM	1.247	1626
5(-)	Staphylococcus muscae DSM 7068T DSM	1.242	1294
6(-)	Streptococcus massiliensis DSM 18628T DSM	1.239	313439
7(-)	Enterobacter cloacae 20105_2 CHB	1.216	550
8(-)	Providencia rettgeri CCM 5618 CCM	1.177	587
9(-)	Streptococcus sanguinis CIP 55_128T CIP	1.176	1305
10(-)	Pseudomonas antarctica DSM 15318T HAM	1.173	219572

Analyte18

Analyte Name: B6

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus aureus ssp aureus DSM 3463 DSM	2.251	46170
2(++)	Staphylococcus aureus ATCC 33862 THL	2.192	1280
3(++)	Staphylococcus aureus ATCC 29213 THL	2.138	1280
4(++)	Staphylococcus aureus ssp aureus DSM 346 DSM	2.095	46170
5(++)	Staphylococcus aureus ssp aureus DSM 4910 DSM	2.091	46170
6(++)	Staphylococcus aureus ATCC 33591 THL	2.075	1280
7(+)	Staphylococcus aureus ATCC 25923 THL	1.954	1280
8(+)	Staphylococcus aureus ssp aureus DSM 11822 DSM	1.861	46170

Analyte19

Analyte Name: B7

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Enterobacter asburiae DSM 17506T DSM	2.255	61645
2(++)	Enterobacter kobei DSM 13645T DSM	2.192	208224
3(++)	Enterobacter cloacae MB11506_1 CHB	2.076	550
4(++)	Enterobacter ludwigii DSM 16688T DSM	2.033	299767
5(+)	Enterobacter cloacae 13159_1 CHB	1.954	550
6(+)	Enterobacter cloacae DSM 6234 DSM	1.95	550
7(+)	Enterobacter cloacae 20105_2 CHB	1.889	550
8(+)	Enterobacter cloacae MB_5277_05 THL	1.874	550
9(+)	Enterobacter hormaechei ssp hormaechei DSM 12409T DSM	1.825	301105
10(+)	Enterobacter cloacae DSM 30062 DSM	1.808	550

Analyte20

Analyte Name: B8

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus aureus ssp aureus DSM 3463 DSM	2.215	46170
2(++)	Staphylococcus aureus ATCC 33862 THL	2.177	1280
3(++)	Staphylococcus aureus ssp aureus DSM 346 DSM	2.172	46170
4(++)	Staphylococcus aureus ATCC 33591 THL	2.166	1280
5(++)	Staphylococcus aureus ssp aureus DSM 4910 DSM	2.127	46170
6(++)	Staphylococcus aureus ATCC 29213 THL	2.116	1280
7(++)	Staphylococcus aureus ssp aureus DSM 20232 DSM	2	46170
8(+)	Staphylococcus aureus ssp aureus DSM 11822 DSM	1.969	46170
9(+)	Staphylococcus aureus ATCC 25923 THL	1.942	1280

Analyte21

Analyte Name: B9

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.828

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus aureus ssp aureus DSM 3463 DSM	2.189	46170
2(++)	Staphylococcus aureus ATCC 33862 THL	2.082	1280
3(++)	Staphylococcus aureus ssp aureus DSM 4910 DSM	2.057	46170
4(++)	Staphylococcus aureus ATCC 33591 THL	2.03	1280
5(++)	Staphylococcus aureus ATCC 29213 THL	2.011	1280
6(++)	Staphylococcus aureus ssp aureus DSM 346 DSM	2.006	46170
7(+)	Staphylococcus aureus ssp aureus DSM 20491 DSM	1.95	46170
8(+)	Staphylococcus aureus ATCC 25923 THL	1.905	1280
9(+)	Staphylococcus aureus ssp aureus DSM 20232 DSM	1.858	46170

Analyte22

Analyte Name: B10

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(++)	Staphylococcus aureus ssp aureus DSM 346 DSM	2.185	46170
2(++)	Staphylococcus aureus ssp aureus DSM 4910 DSM	2.154	46170
3(++)	Staphylococcus aureus ATCC 25923 THL	2.153	1280
4(++)	Staphylococcus aureus ssp aureus DSM 20232 DSM	2.128	46170
5(++)	Staphylococcus aureus ssp aureus DSM 11822 DSM	2.062	46170
6(++)	Staphylococcus aureus ATCC 33591 THL	2.042	1280
7(++)	Staphylococcus aureus ATCC 29213 THL	2.041	1280
8(+)	Staphylococcus aureus ATCC 33862 THL	1.914	1280
9(+)	Staphylococcus aureus ssp aureus DSM 20491 DSM	1.886	46170

Analyte23

Analyte Name: B11

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(-)	no peaks found	< 0	-
2(-)	no peaks found	< 0	-
3(-)	no peaks found	< 0	-
4(-)	no peaks found	< 0	-
5(-)	no peaks found	< 0	-
6(-)	no peaks found	< 0	-
7(-)	no peaks found	< 0	-
8(-)	no peaks found	< 0	-
9(-)	no peaks found	< 0	-
10(-)	no peaks found	< 0	-

Analyte24

Analyte Name: B12

Analyte Description:

Analyte ID:

Analyte Creation Date/Time: 2010-07-26 18:16:55.843

Applied MSP Library(ies):

Applied Taxonomy Tree: Bruker Taxonomy

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (++)	Escherichia coli ATCC 25922 THL	2.281	562
2 (++)	Escherichia coli MB11464_1 CHB	2.14	562
3 (++)	Escherichia coli DH5alpha BRL	2.12	562
4 (++)	Escherichia coli ATCC 25922 CHB	2.036	562
5 (++)	Escherichia coli DSM 30083T HAM	2.003	562
6 (+)	Escherichia coli Nissl VML	1.955	562

4. Super Optimal Growth Media (S.O.C)

2% Tryptone

0.5% Yeast Extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

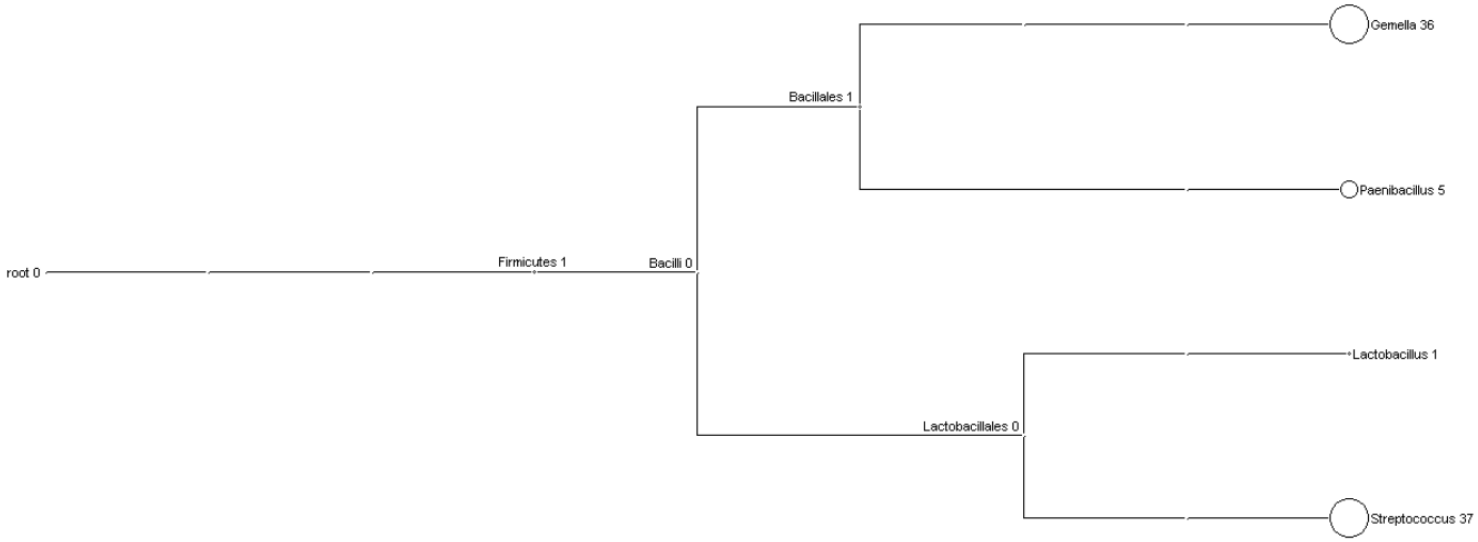
10 mM $MgSO_4$

20 mM Glucose

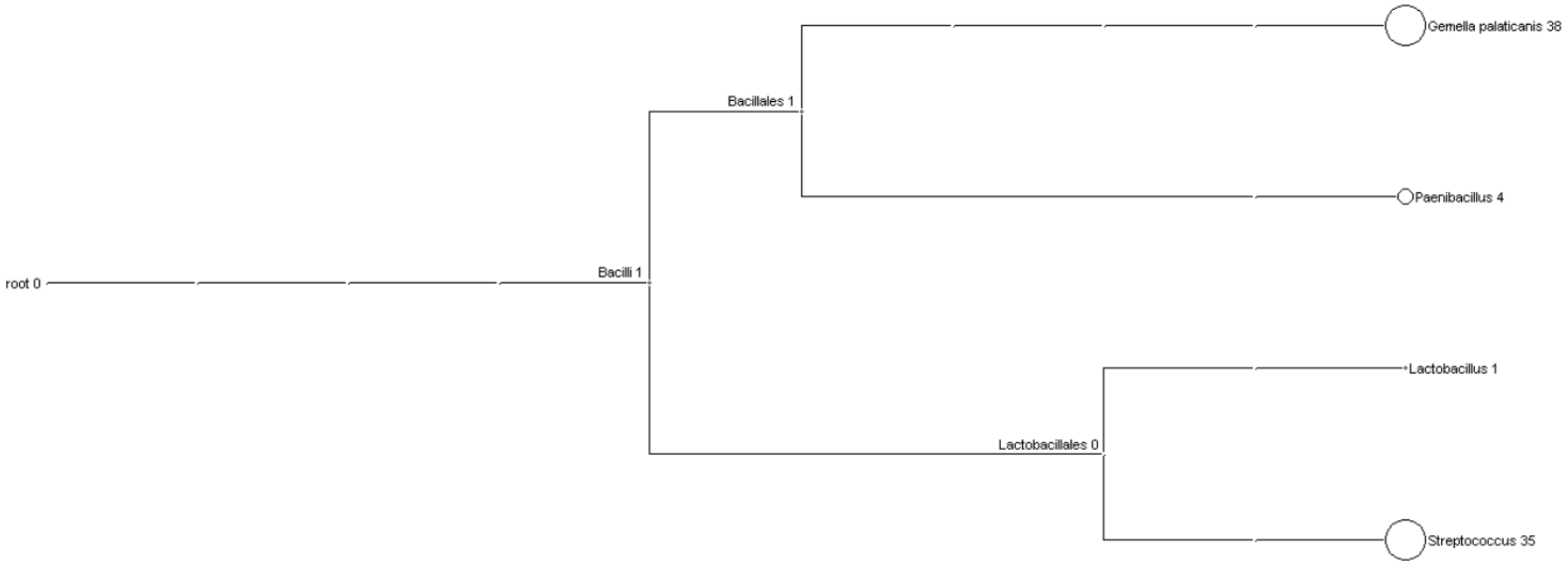
5. Phylogenetic trees

5.1. Specific pathogen free (SPF) C3H to germ free (GF) C3H Transmission experiments

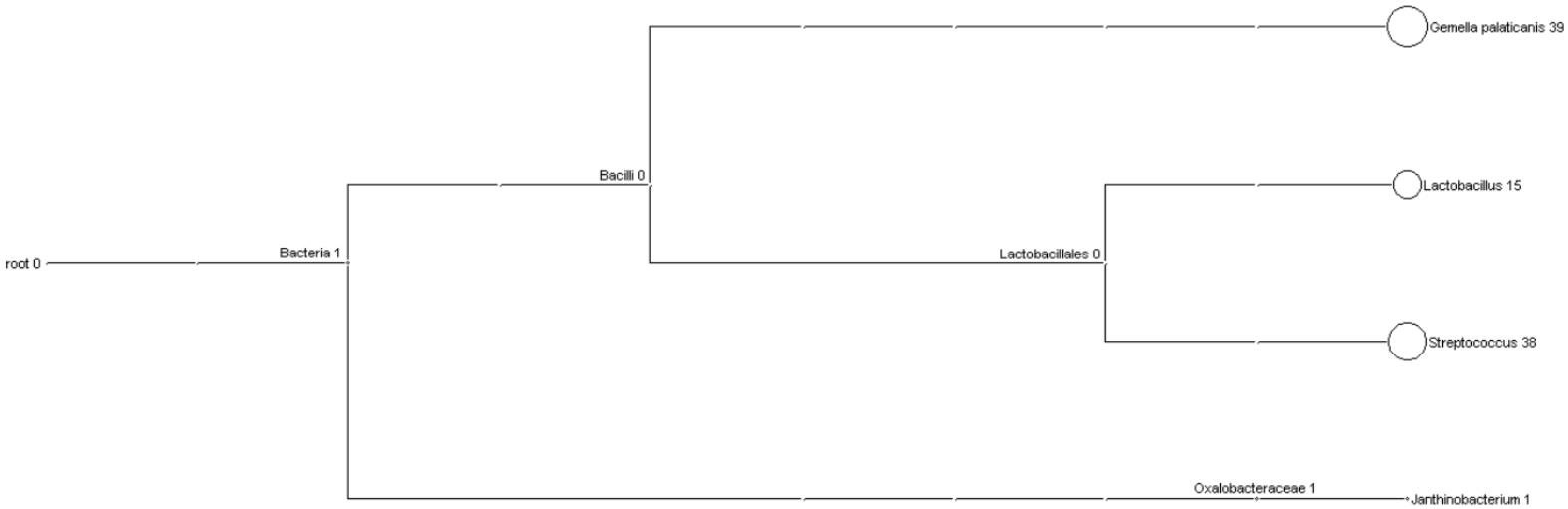
5.1.1. SPF 1 – Specific Pathogen Free (conventional) mice cage 1 (n=3)



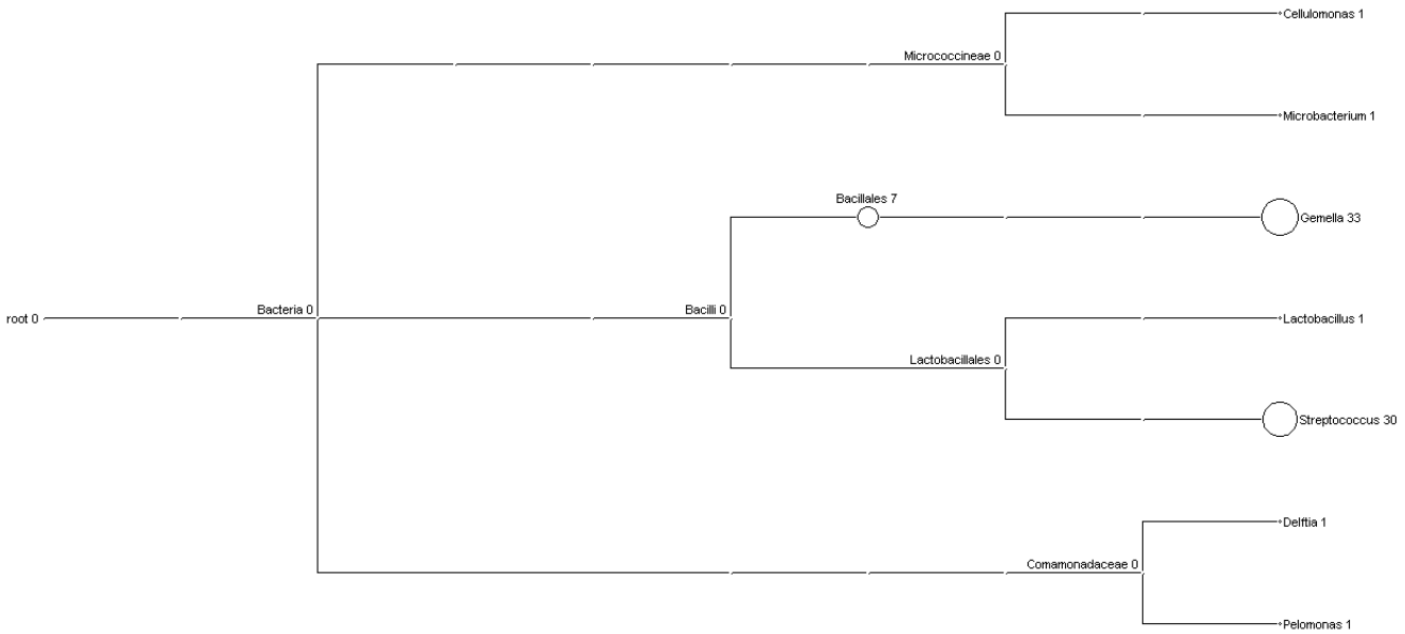
5.1.2. SPF 2 – Specific Pathogen Free (conventional) mice cage 2 (n=2)



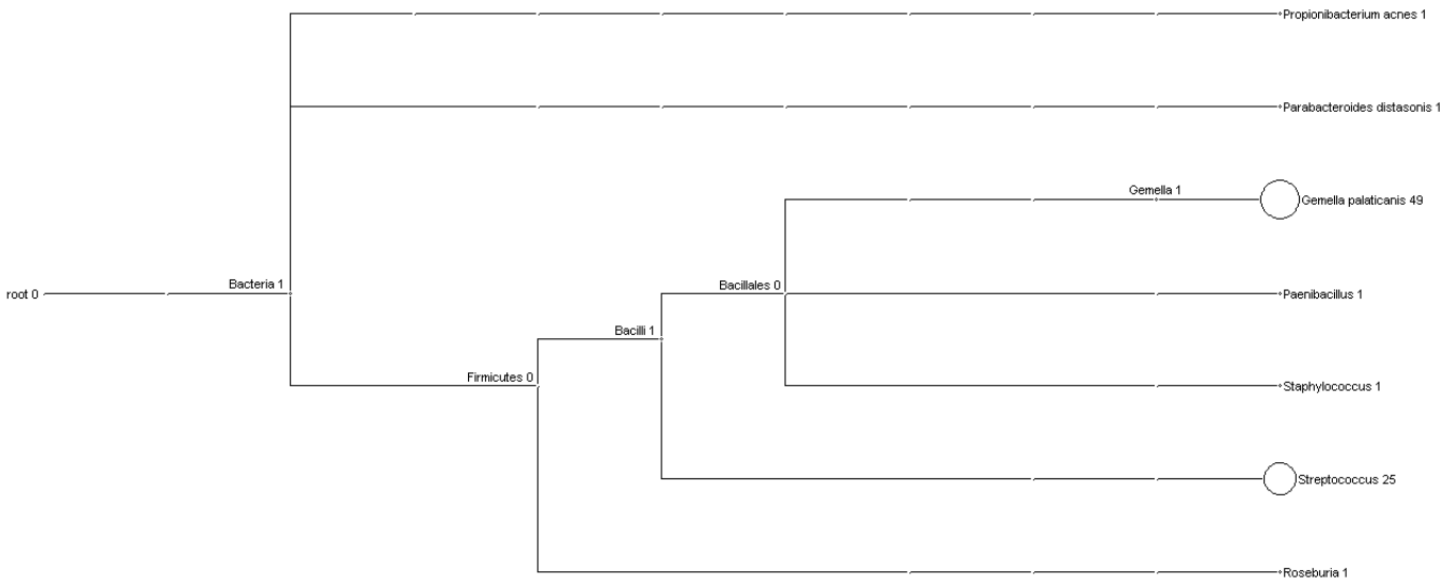
5.1.3. CNV1 – Previously germ free (conventionalised) mice cage 1 (n=2)



5.1.4. CNV 2 – Previously germ free (conventionalised) mice cage 2 (n=2)

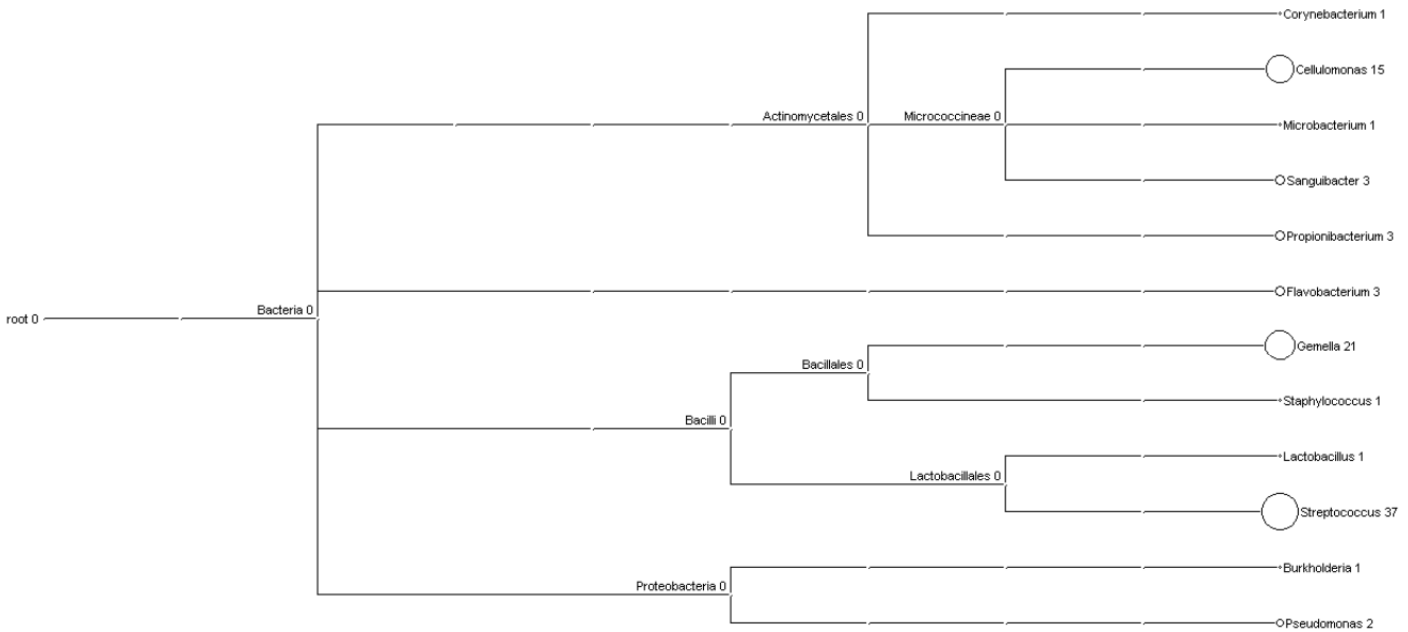


5.1.5. CNV 3 – Previously germ free (conventionalised) mice cage 3 (n=2)

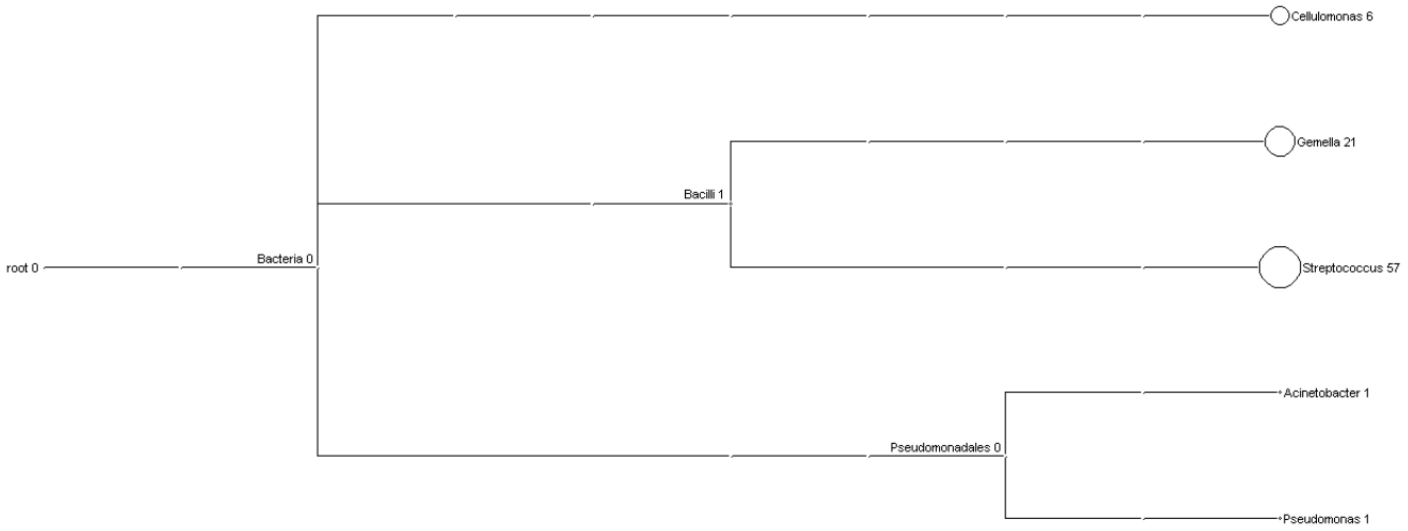


5.2. *Porphyromonas gingivalis* challenge experiments

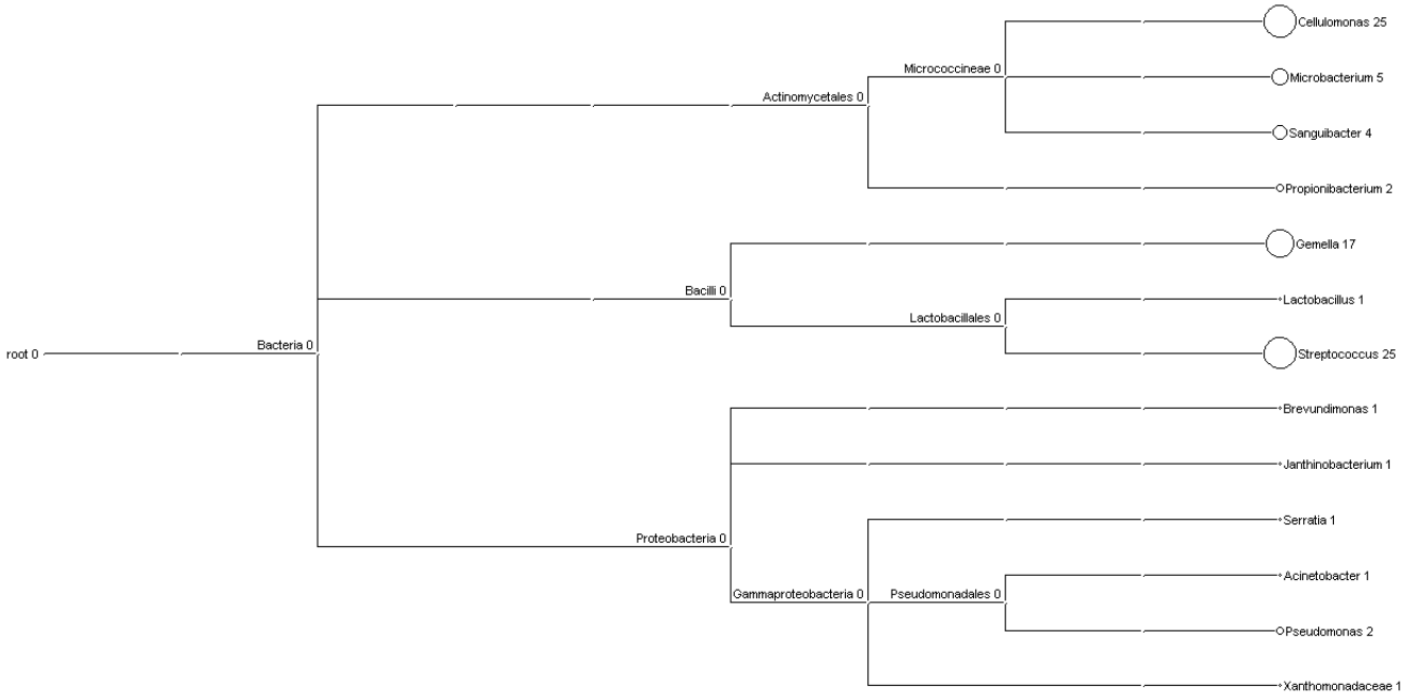
5.2.1. Cage 1 SPF control mice (n=3)



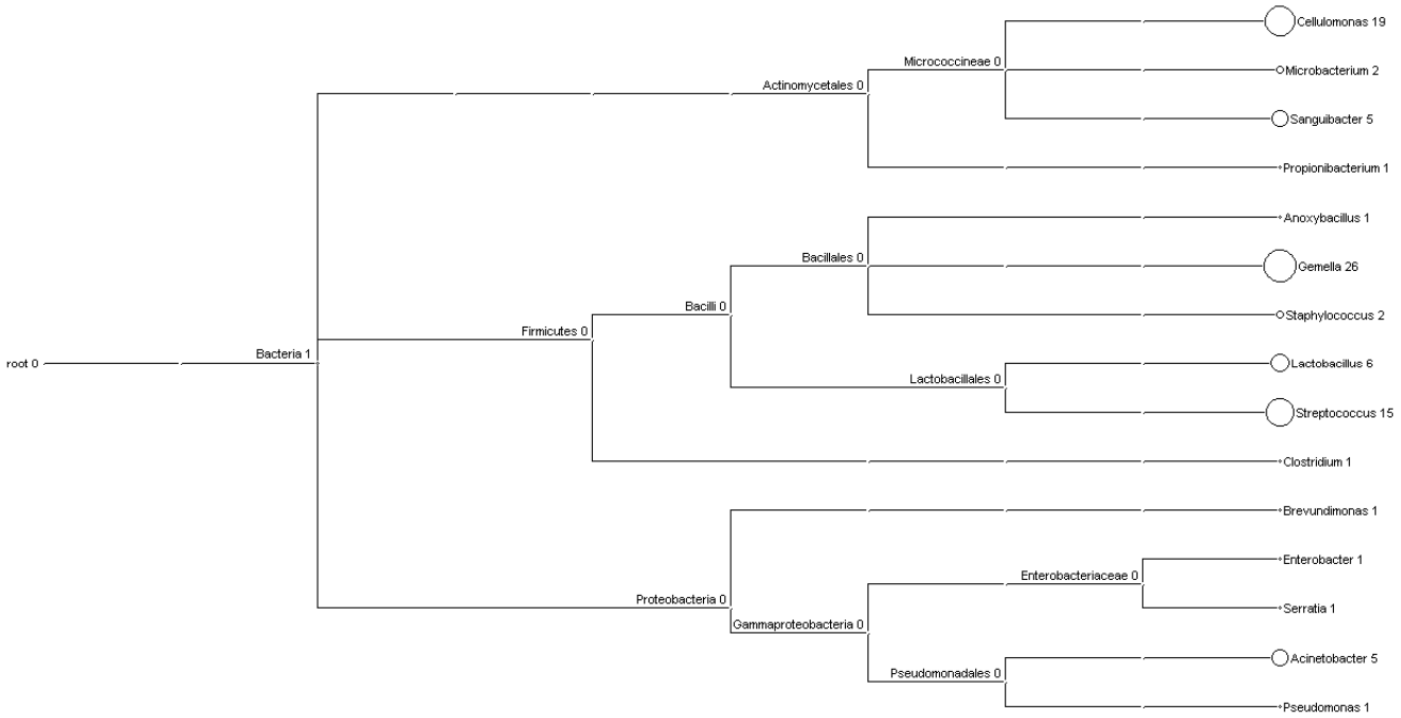
5.2.2. Cage 2 SPF control mice (n=3)



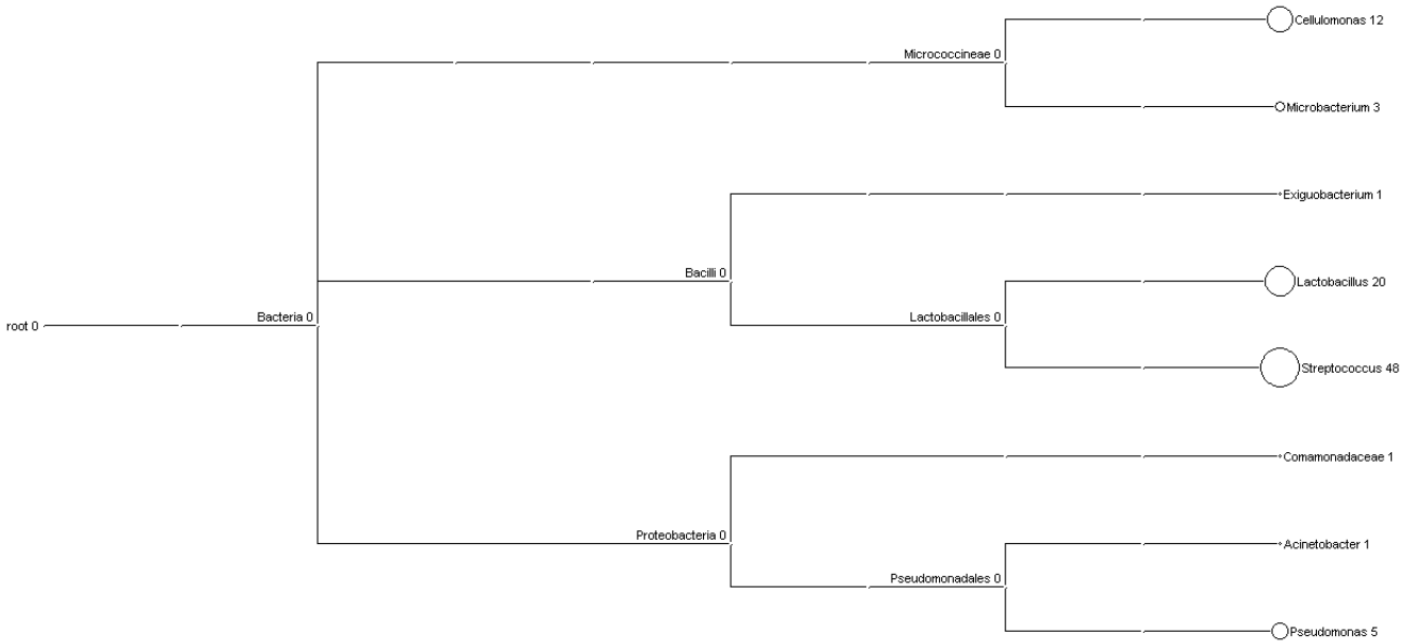
5.2.3. Cage 3 SPF control mice (n=4)



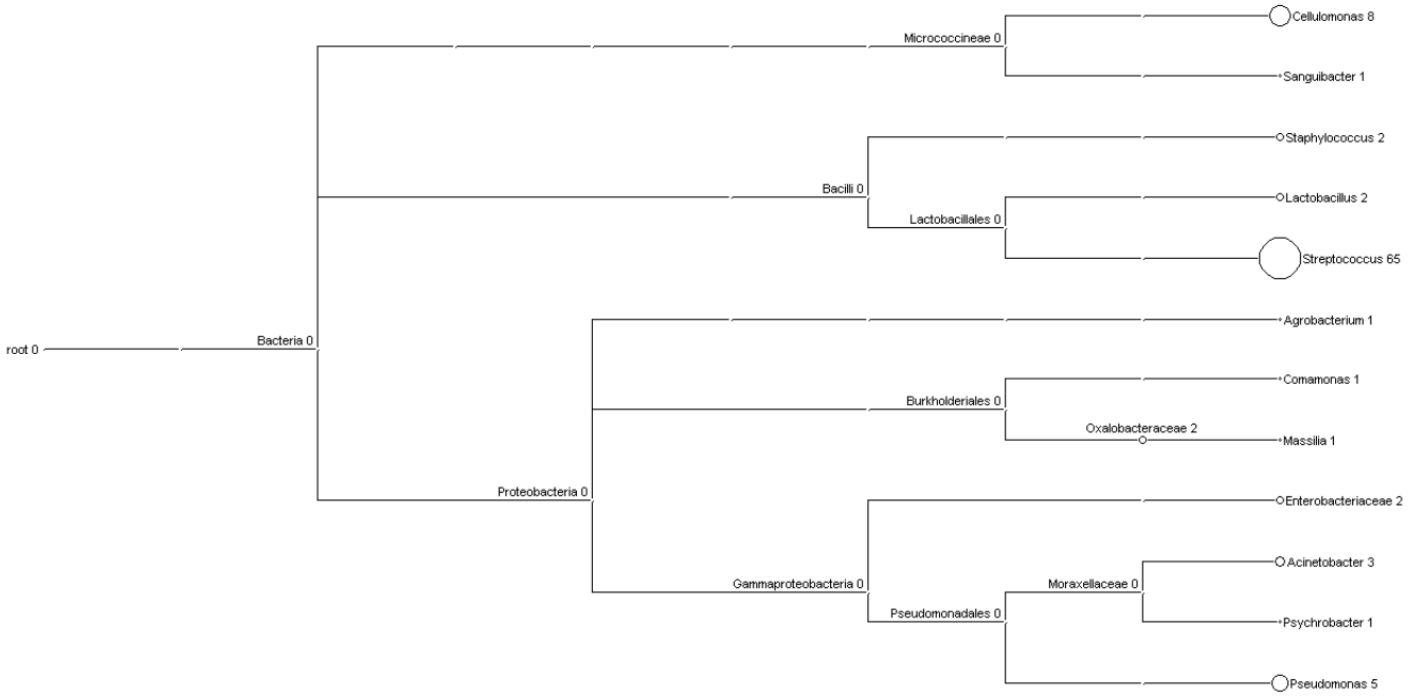
5.2.4. Cage 4 SPF control mice (n=2)



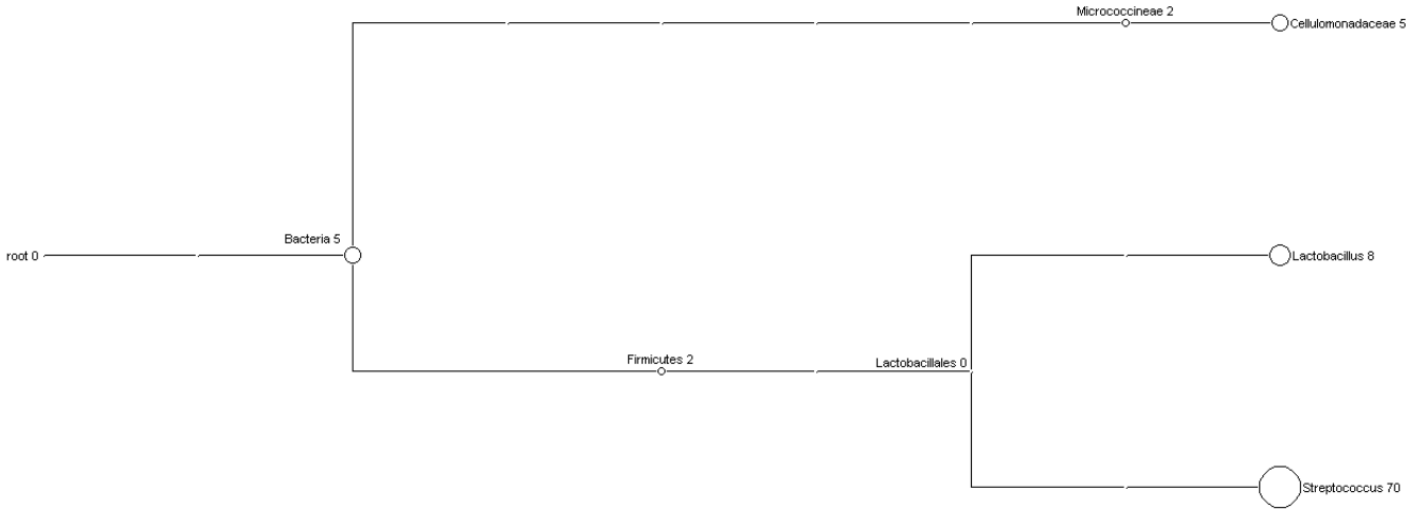
5.2.5. Cage 5 SPF mice given antibiotics only (n=3)



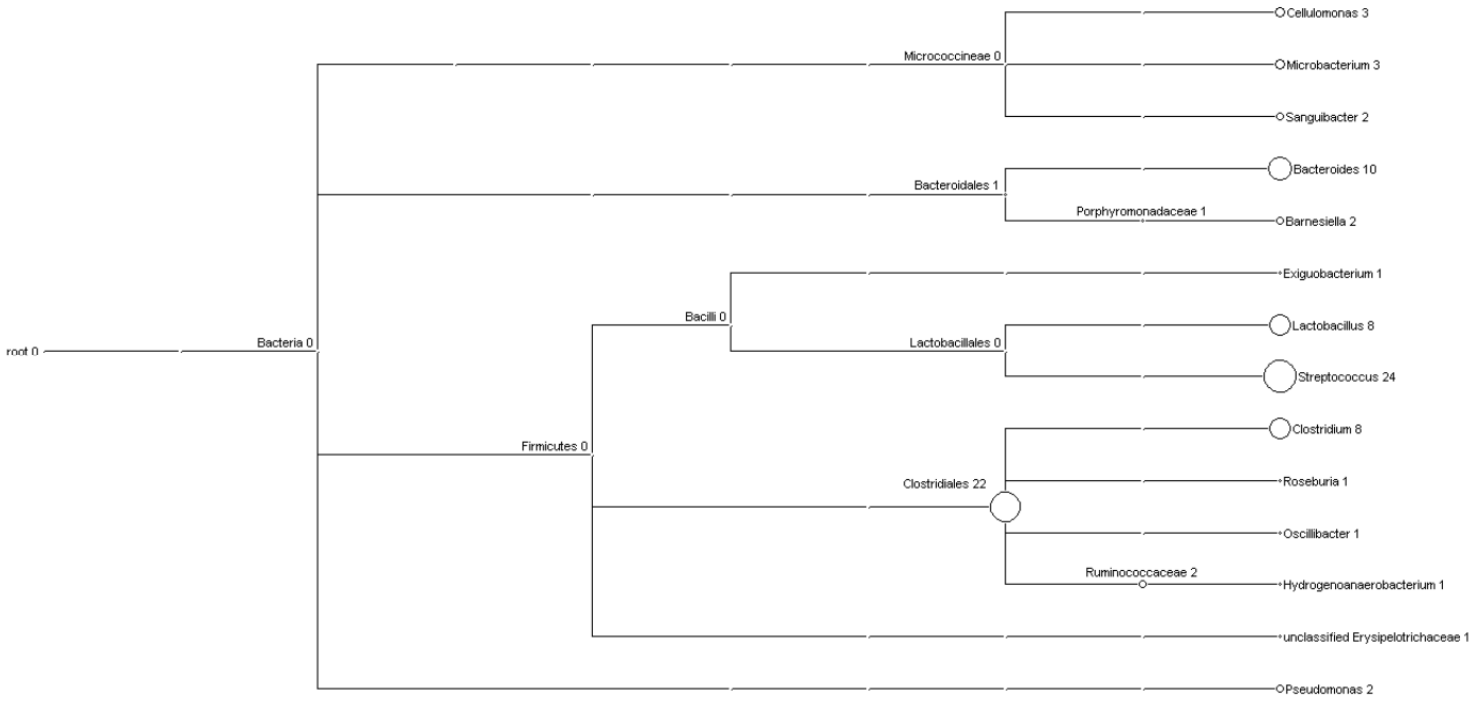
5.2.6. Cage 6 SPF mice given antibiotics only (n=3)



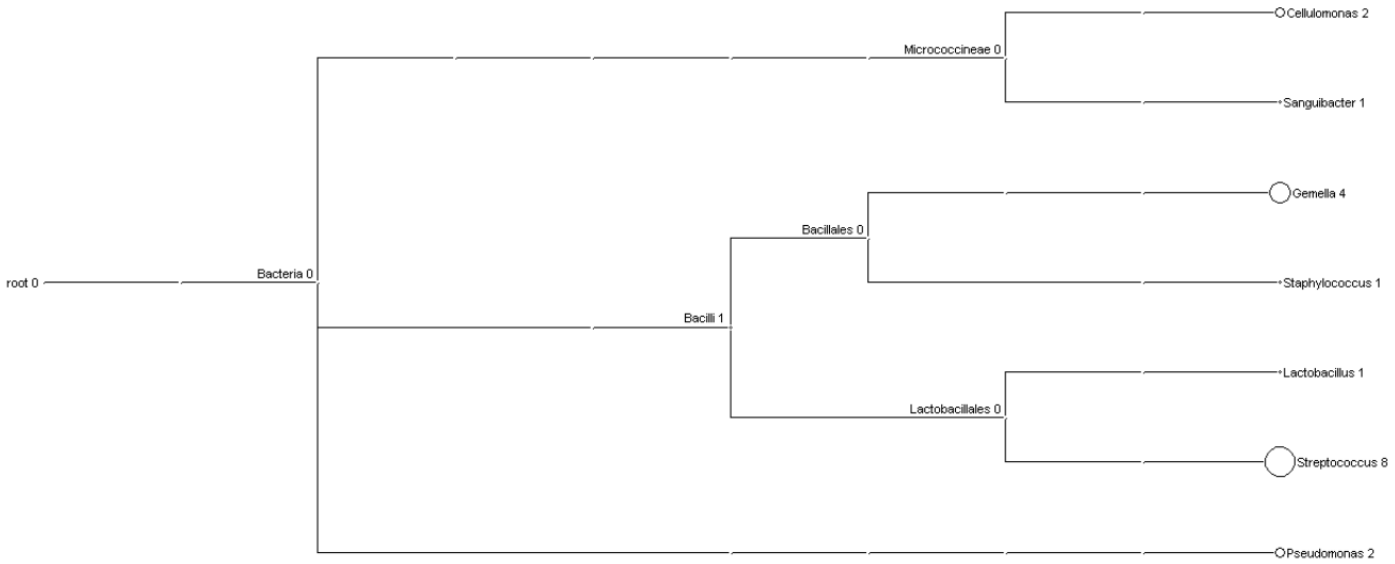
5.2.7. Cage 7 SPF mice given antibiotics only (n=4)



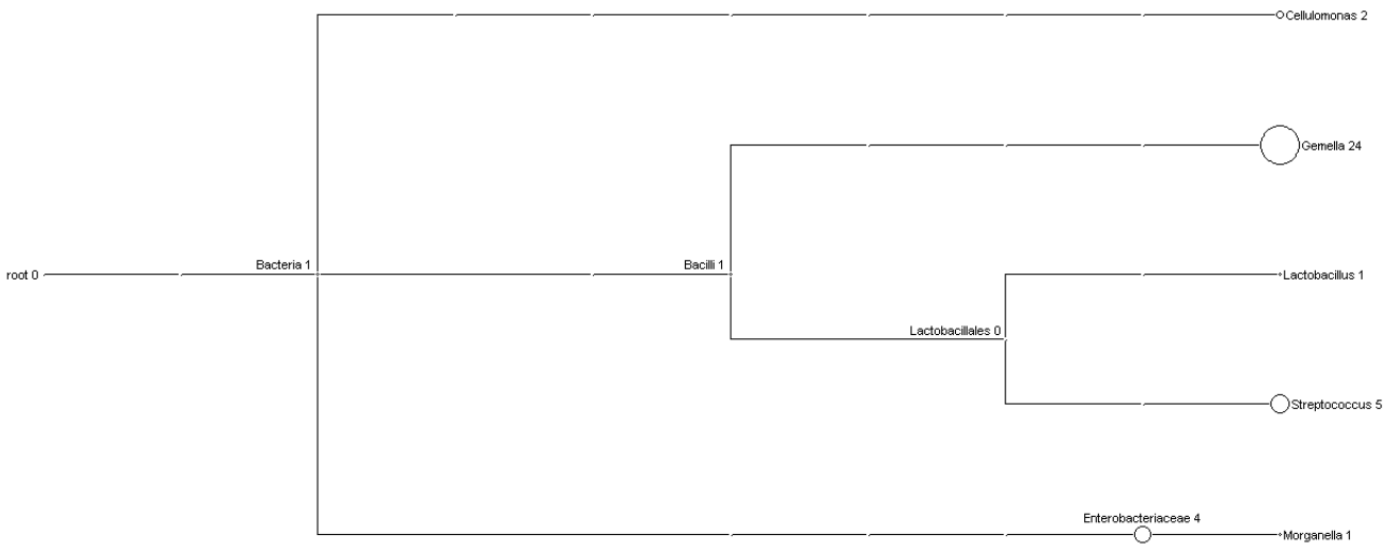
5.2.8. Cage 8 SPF mice given antibiotics only (n=2)



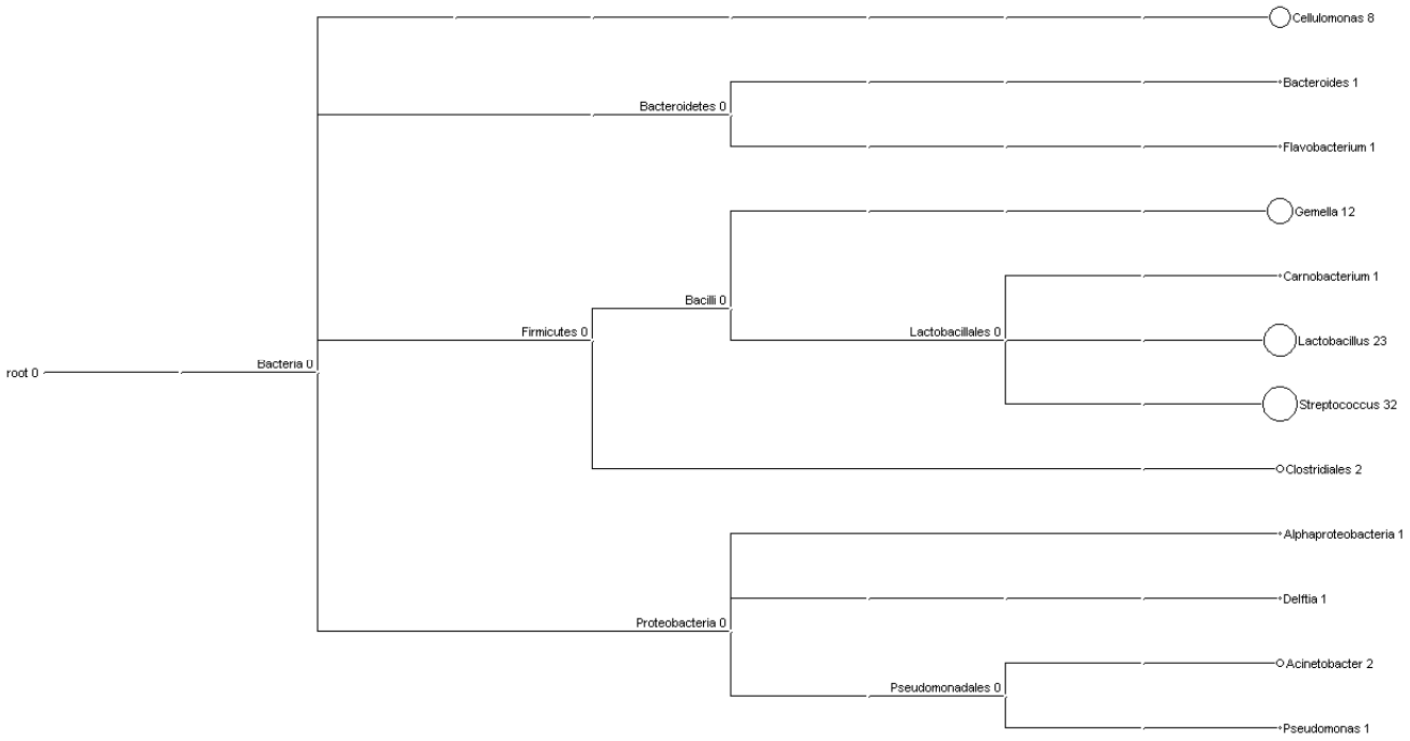
5.2.9. Cage 9 SPF mice inoculated with *P. gingivalis* W50 only (n=4)



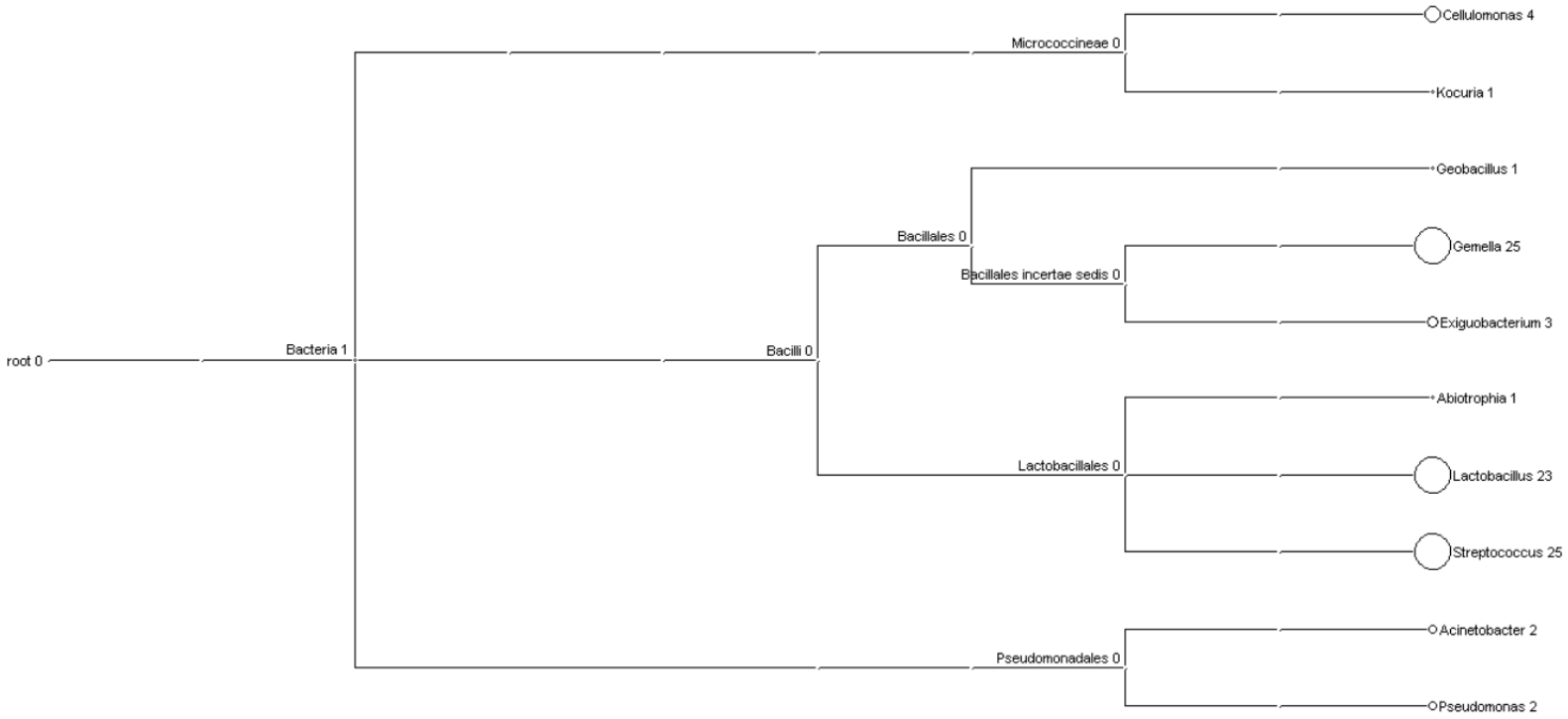
5.2.10. Cage 10 SPF mice inoculated with *P. gingivalis* W50 only (n=3)



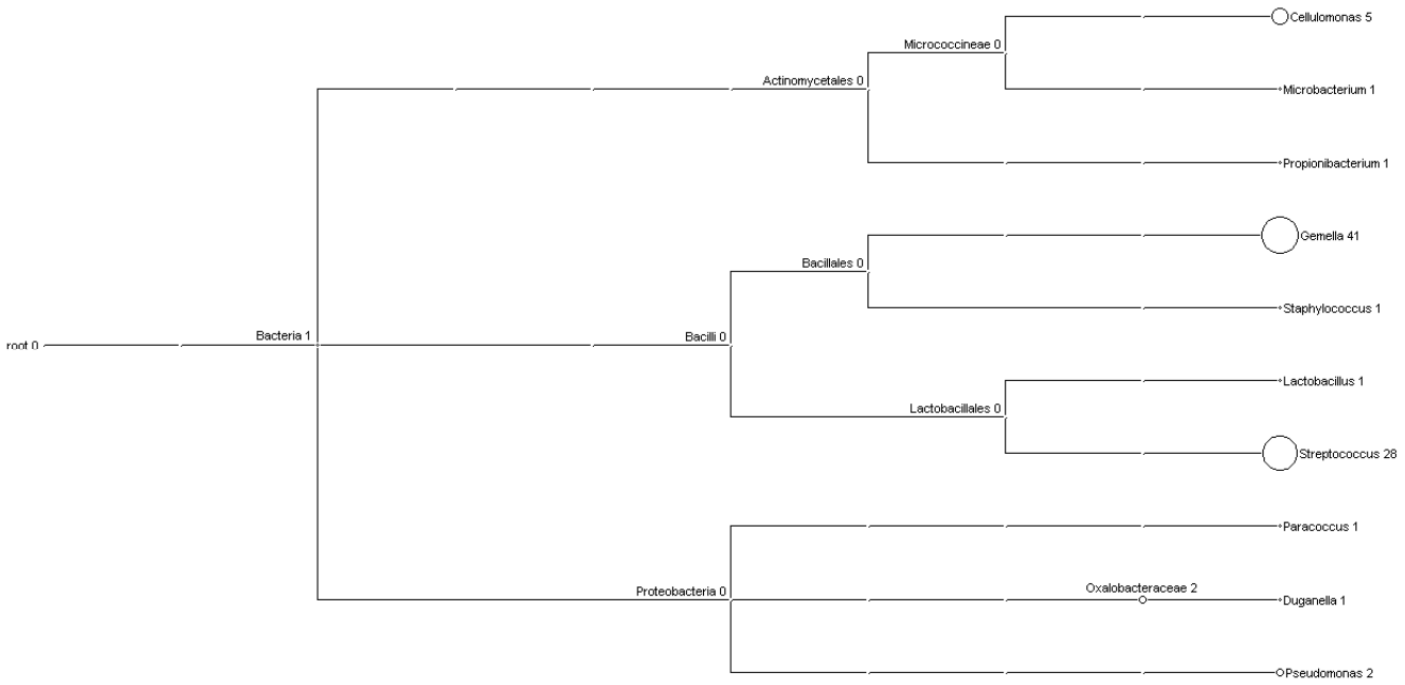
5.2.11. Cage 11 SPF mice inoculated with *P. gingivalis* W50 only (n=4)



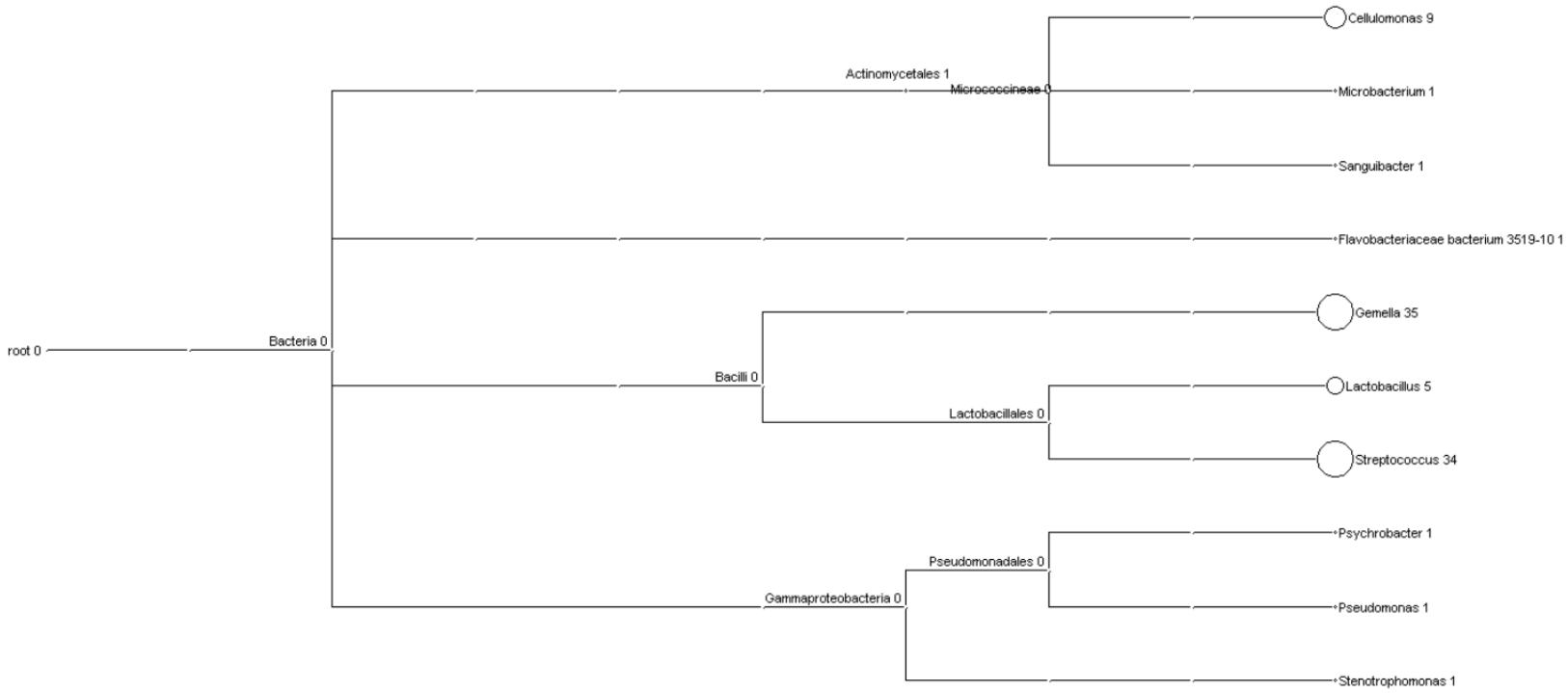
5.2.12. Cage 12 SPF mice inoculated with *P. gingivalis* W50 only (n=2)



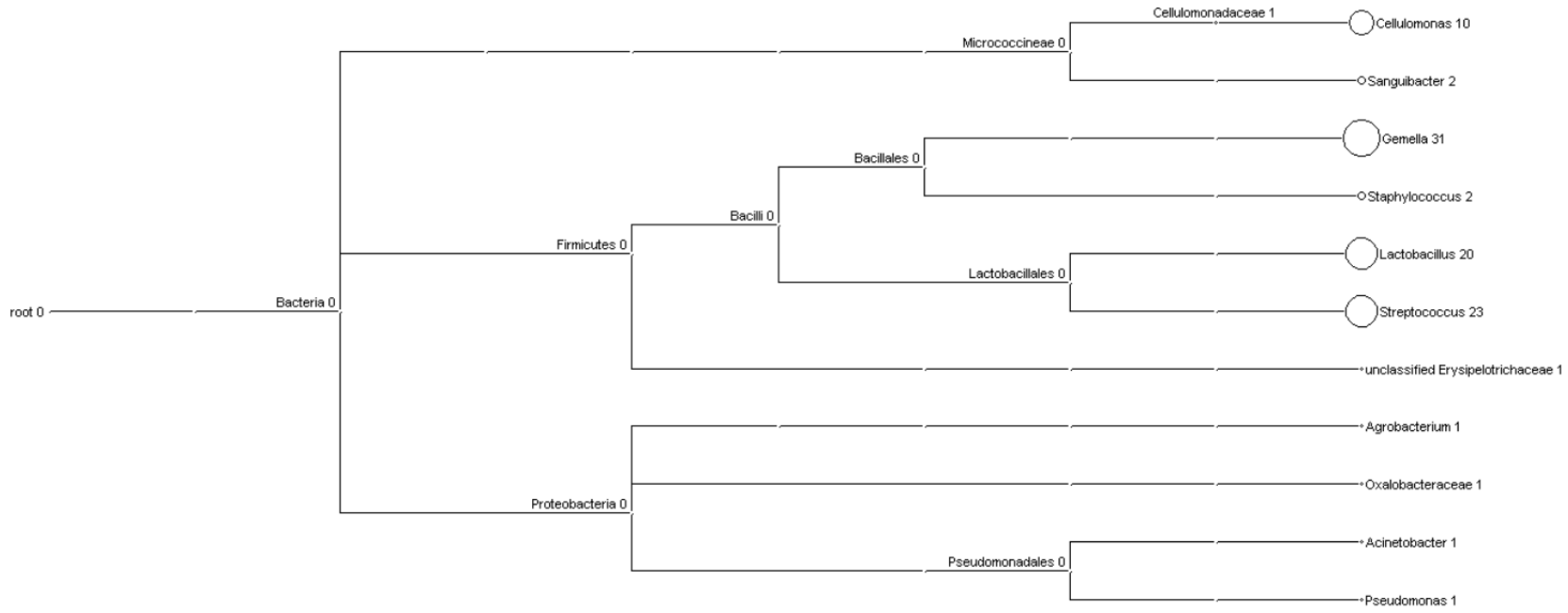
5.2.13. Cage 13 SPF given antibiotics and inoculated with *P. gingivalis* W50 (n=3)



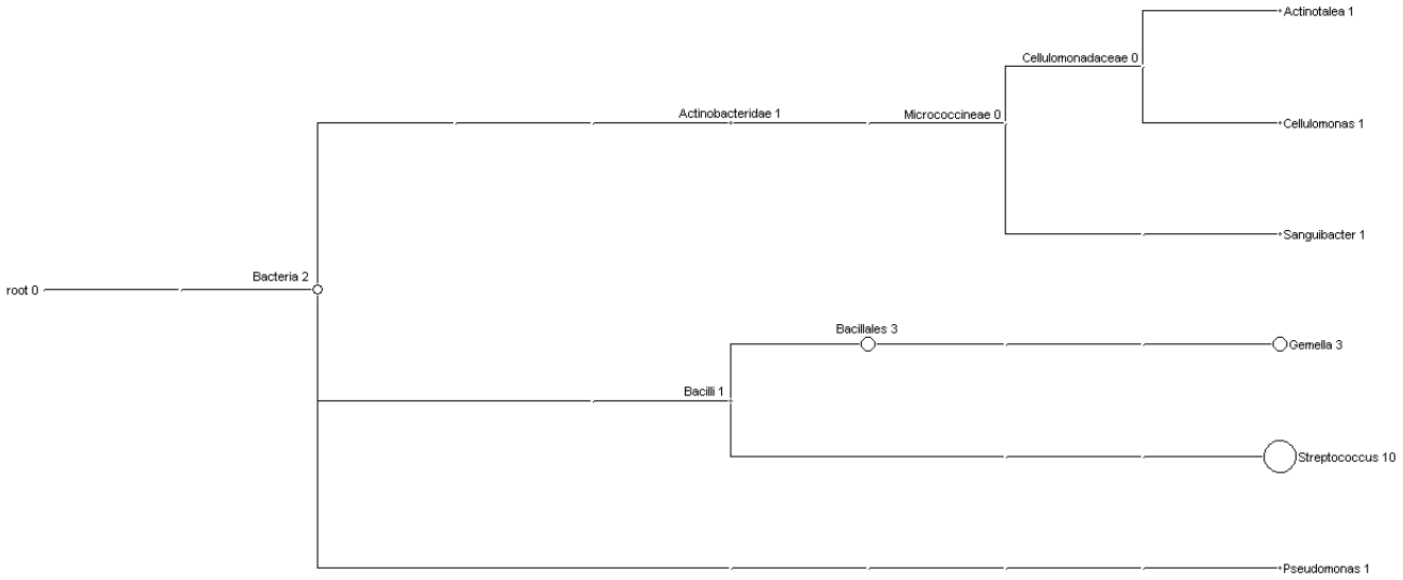
5.2.14. Cage 14 SPF given antibiotics and inoculated with *P. gingivalis* W50 (n=3)



5.2.15. Cage 15 SPF given antibiotics and inoculated with *P. gingivalis* W50 (n=5)

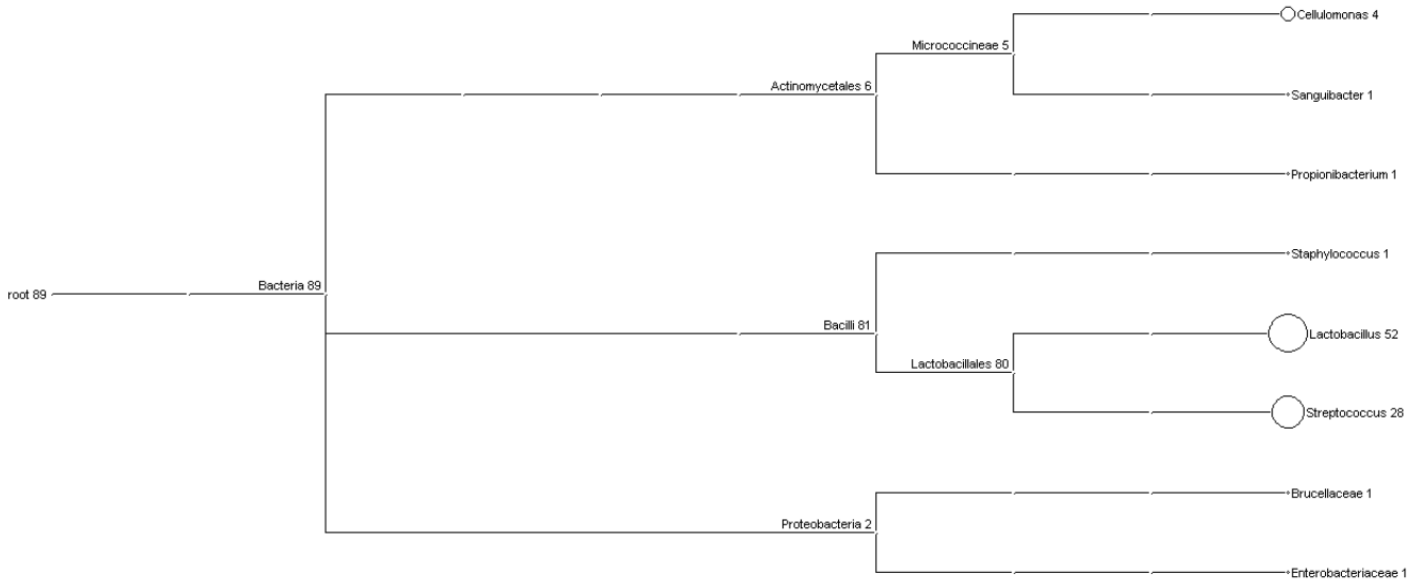


5.2.16. Cage 16 SPF given antibiotics and inoculated with *P. gingivalis* W50 (n=2)

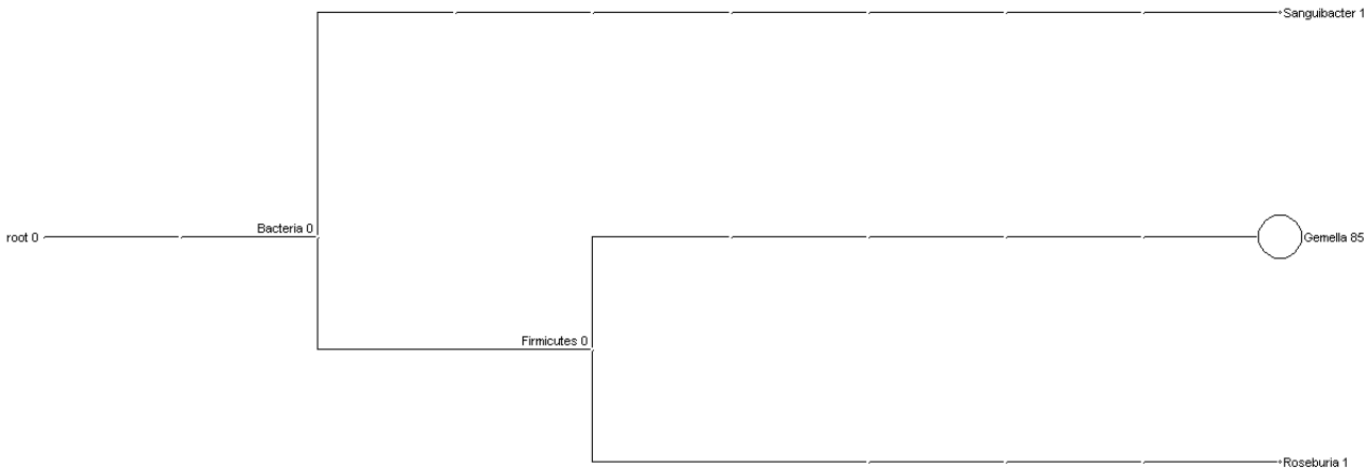


5.3. Specific pathogen free (SPF) Balb/c to germ free (GF) C3H transmission experiments

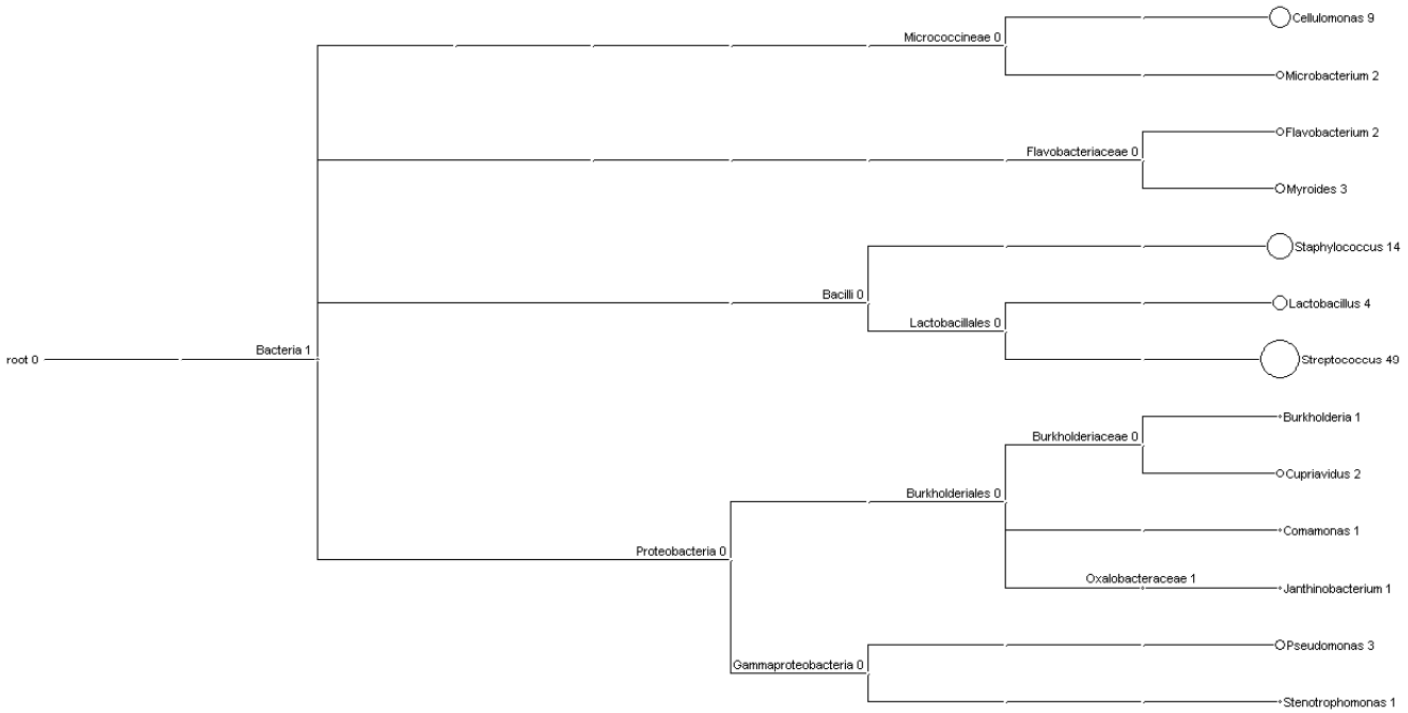
5.3.1. Balb/c specific pathogen free (SPF) cage 1 (n=2)



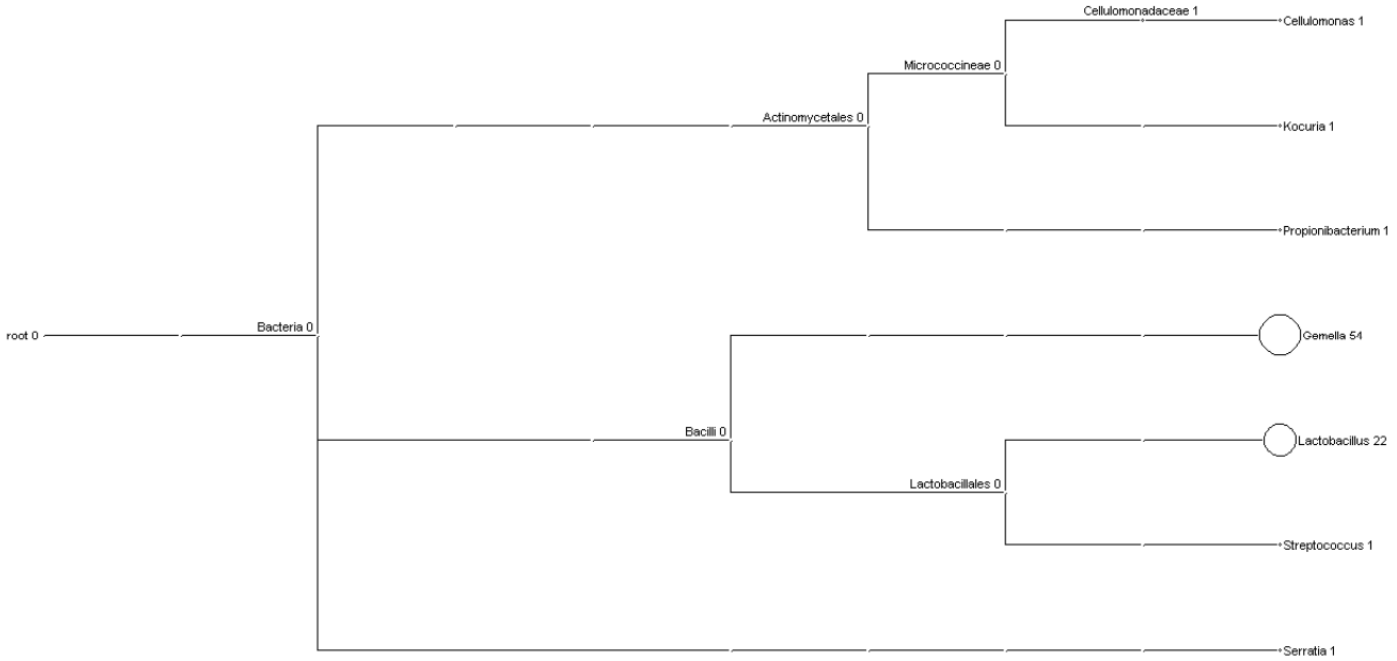
5.3.2. Balb/c specific pathogen free (SPF) cage 3 (n=2)



5.3.3. Previously germ free (conventionalised) C3H mice cage 1 (n=2)

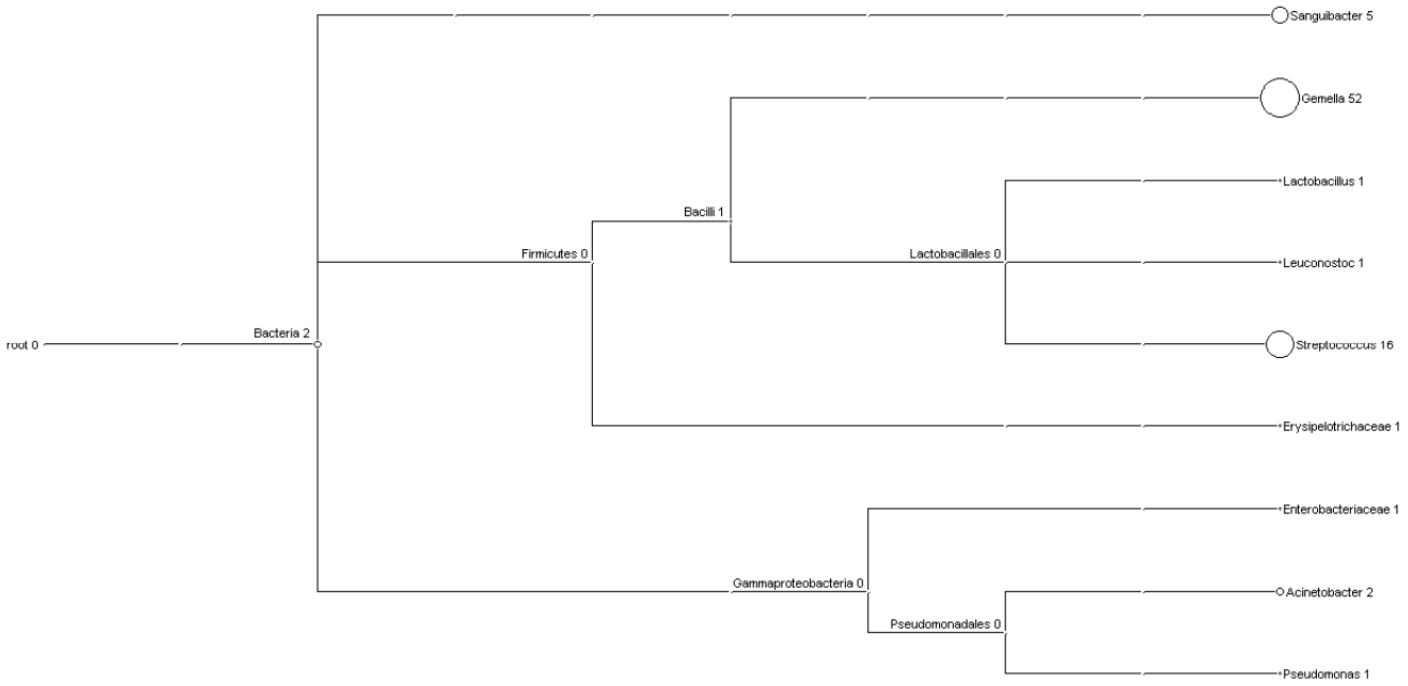


5.3.4. Previously germ free (conventionalised) C3H mice cage 3 (n=2)

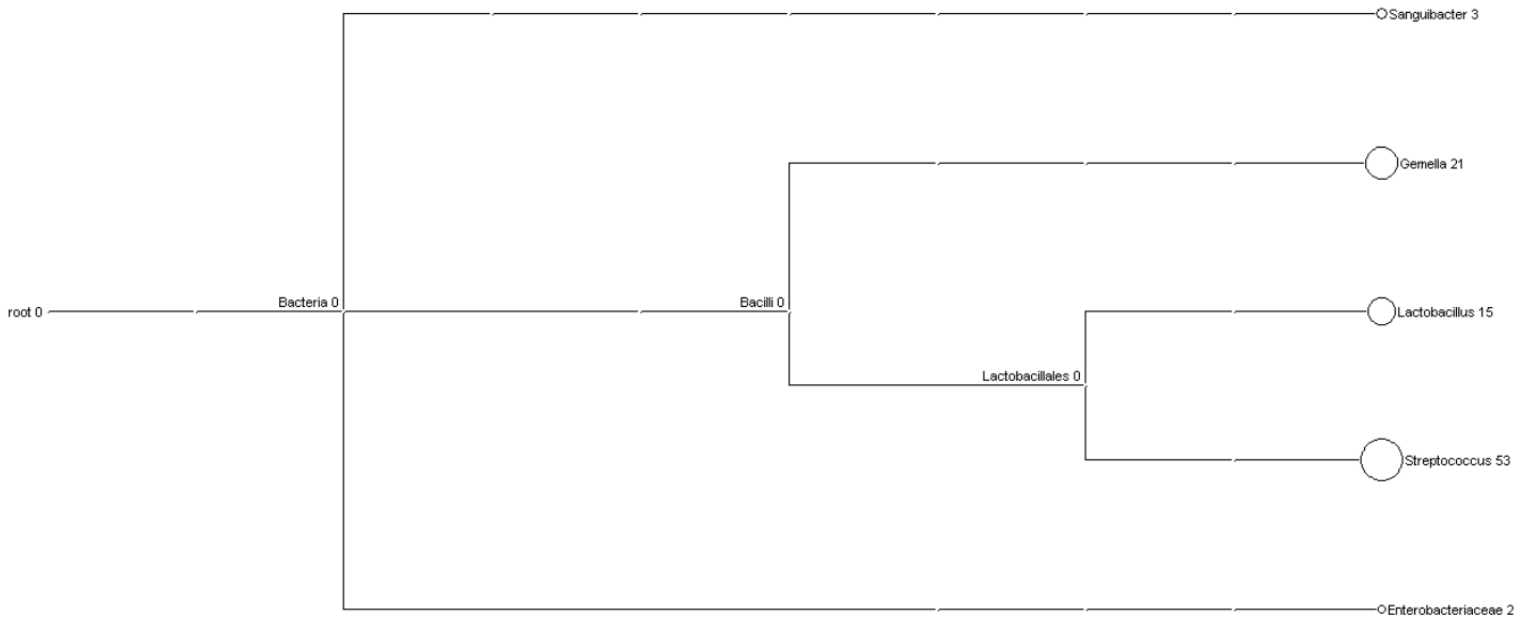


5.4. Specific pathogen free (SPF) CXCR2-/- to germ free (GF) C3H transmission experiments

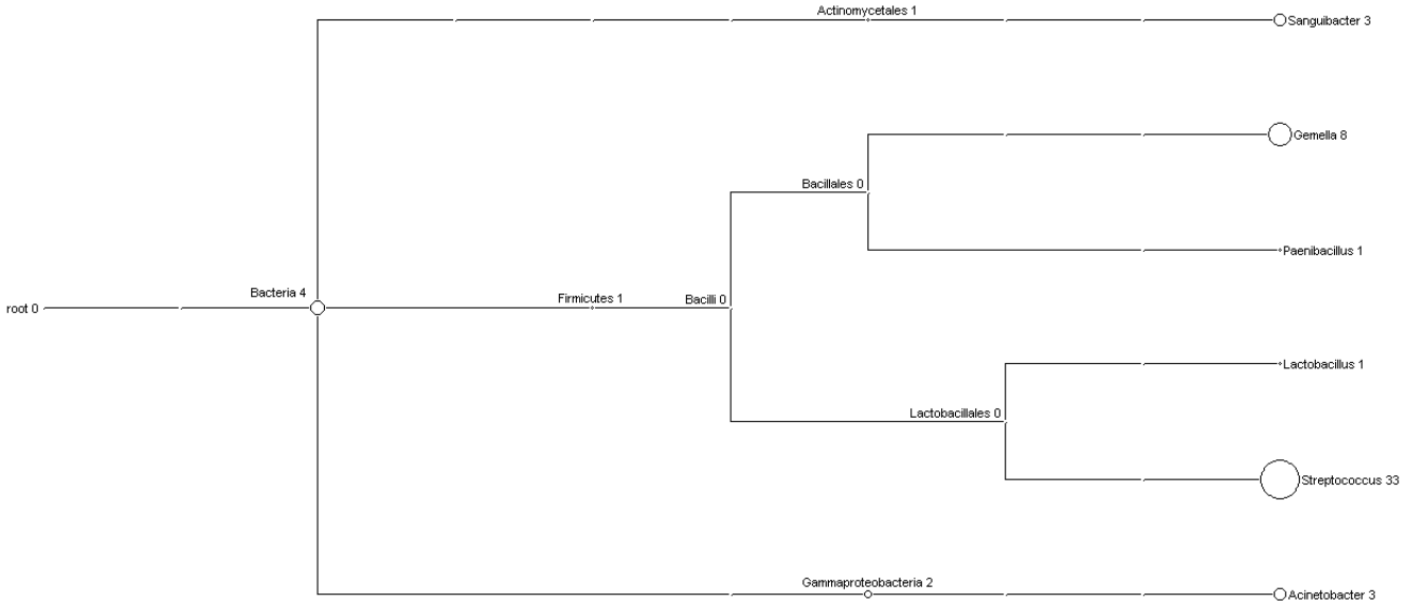
5.4.1. Specific pathogen free (SPF) CXCR2-/- cage 1 (n=2)



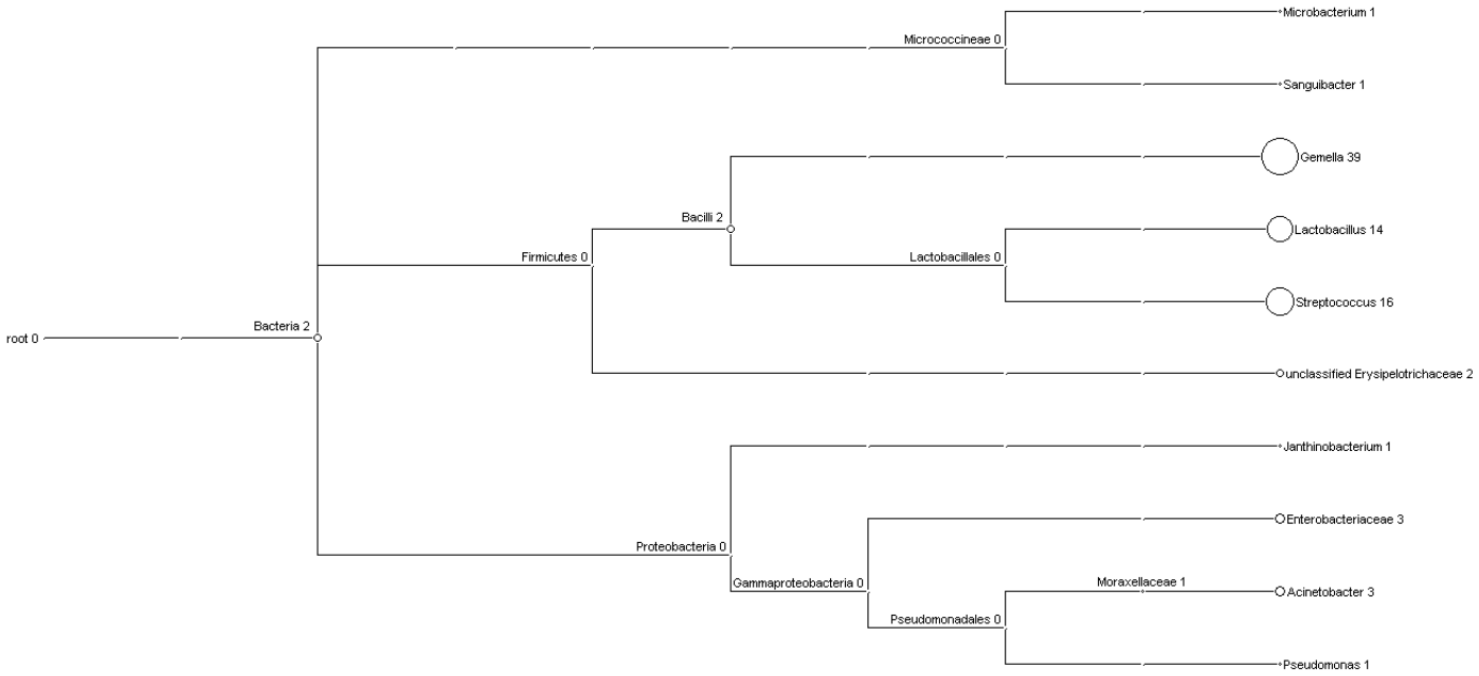
5.4.2. Specific pathogen free (SPF) CXCR2-/- cage 2 (n=2)



5.4.3. Previously germ free (conventionalised) C3H mice cage 1 (n=4)

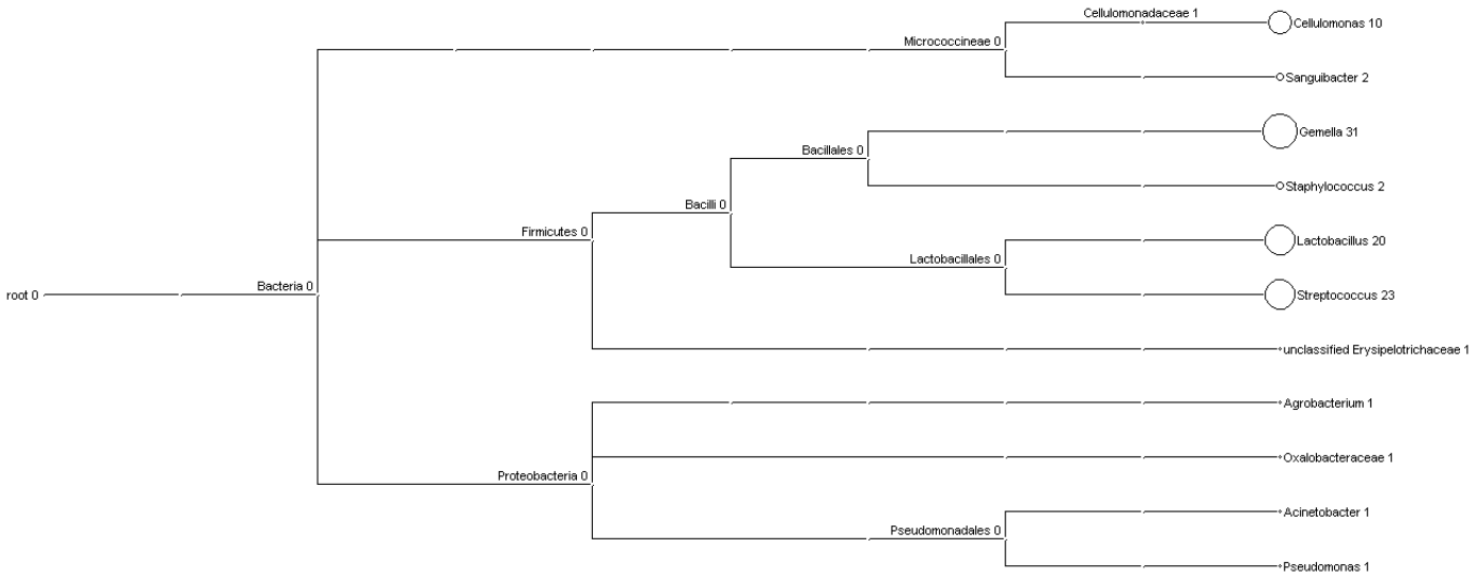


5.4.4. Previously germ free (conventionalised) C3H mice cage 2 (n=4)

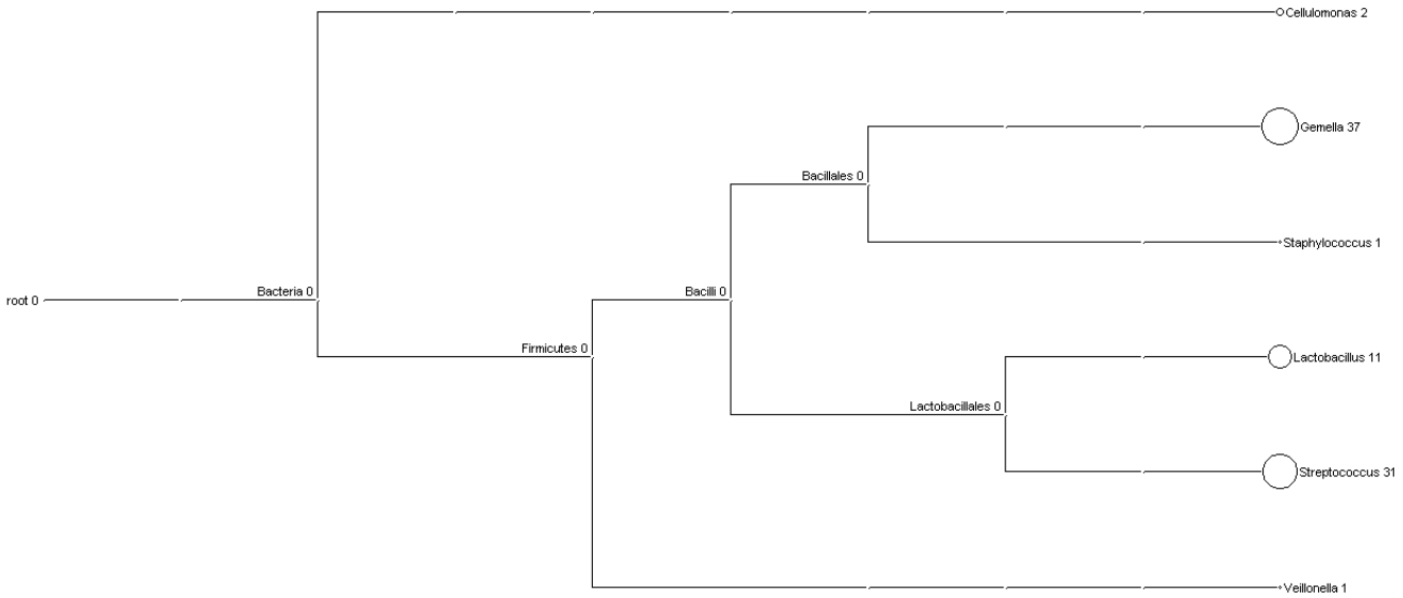


5.5. *Porphyromonas gingivalis* altered flora in SPF C3H to germ free C3H mice transmission experiment

5.5.1. Specific pathogen free C3H mice following inoculation with P. gingivalis (n=5)



5.5.2. Previously germ free (altered flora) C3H mice (n=4)



6 Representative Sequences

6.1. Isolates from cultured bacteria

6.1.1. *Gemella sp*

Range 1335/1424 Identity 97% Sample Aero 2

GGACGACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAAGTTTAAGAGATGCTTGCAT
CACTTGAACTTAGCGGCGAACGGGTGAGTAACACGTAAAGAACCTGCCTCATAGACTGGGAC
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TCGGTCAAGCTGACACTATGAGATGGCTTTGCGGTGCATTAGCTAGTTGGTAGGGTAAAGGCC
TACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
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CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG
CGCAGGTGGTTTAATAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAA
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GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATACAGTGAAGATTTTAGAG
ATAGAATTGTTCTTACCTTTGGTAAGACACTGATACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATATCTAGTTGCCAGCAGTA
AGATGGGGACTCTAGATAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACTTGGGCTACACACGTACTACAATGGATAGGAACAAAGAGAAGCA
AACTCGCGAGAGCAAGCCAACCTCATAAAACTATTCTCAGTTCGGATTGTAGTCTGCAACTCGA
CTACATGAAGCTGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGG
TCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGACGGTGGCCTAACC

6.1.2. *Lactobacillus sp*

Range 1474/1480 Identity 99% Sample Aero 12

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GTTAGAGAAGAAAGTGCGTGAGAGTAACTGTTCACGTTTCGACGGTATCTAACCAGAAAGCC
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GTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCAATAA
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ACGAGTCGCAAGACCGCGAGGTTTAGCAAATCTCTTAAAGCCGTTCTCAGTTCGGATTGTAGG
CTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGG
TGGGGTAACCTTTTGGAGCCAGCCGTCTAAGGTGGGACAGATGATAG

6.1.3. *Streptococcus sp*

Range 1382/1424 Identity 97% Sample Aero 1

CTAGATGACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAAAGAGCTTGCTC
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GCAACTGCATCACTACCAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAAAGGCTCAC
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CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGTG
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ACGGCAAGCAAATCTCTTAAAGCCAGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG
AAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTATTAGGA
GCCAGCCGCCTAAGGTGGGATAGATGAGTGG

6.1.4. *Escherichia/Shigella sp*

Range 1453/1461 Identity 99% Sample Aero 13

TAGATTGACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGAAAGCAGCTTGC
TGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGG
ATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTA
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TAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC
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GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
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ACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGG
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6.1.5. *Staphylococcus sp*

Range 1468/1471 Identity 99% Sample Aero 7

TAGATGACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAAGCTTGCTT
CTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATA
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CGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTT
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CGAGGTCAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACAT
GAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGT
ACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCTTTTAGG
AGCTAGCCGTCGAAGGTGGGACAAATGATGGG

6.1.6. *Enterococcus sp*

Range 1471/1475 Identity 99% Sample AN 18

AGGACGACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTT
GCTCCATCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAG
AAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAA
GTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAG
TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAG
AAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTA
AAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC
ATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAA
ATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCT
GAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCG
CCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TAGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACC
ACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCA
TTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGC
GAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCG
CCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACC
TTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATGG

6.1.7. *Propionibacterium sp*

Range 1269/1272 Identity 99% Sample 3

GTGCTTACCATGCAGTCGAACGGAAAGGCCCTGTCTTTTGTGGGGTGCTCGAGTGGCGAACG
GGTGAGTAACACGTGAGTAACCTGCCCTTGACTTTGGGATAACTTCAGGAAACTGGGGCTAAT
ACCGGATAGGAGCTCCTGCTGCATGGTGGGGGTTGGAAAGTTTCGGCGGTTGGGGATGGACT
CGCGGCTTATCAGCTTGTTGGTGGGGTAGTGGCTTACCAAGGCTTTGACGGGTAGCCGGCCTG
AGAGGGTGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCC
TTCGGGTTGTAAACCGCTTTCGCCTGTGACGAAGCGTGAGTGACGGTAATGGGTAAAGAAGC
ACCGGCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGATTTAT
TGGGCGTAAAGGGCTCGTAGGTGGTTGATCGCGTCGGAAGTGTAATCTTGGGGCTTAACCCT
GAGCGTGCTTTCGATACGGGTTGACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGGAG
CGGTGGAATGCGCAGATATCAGGAGGAACACCAGTGGCGAAGGCGGTTCTCTGGGCCTTTCC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGCTTAGATACCCTGGTAGTCCACGCTG
TAAACGGTGGGTACTAGGTGTGGGGTCCATTCCACGGGTTCCGTGCCGTAGCTAACGCTTTAA
GTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGCCCCGCA
CAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGTAGAACCTTACCTGGGTTTGACATG
GATCGGGAGTGCTCAGAGATGGGTGTGCCTCTTTTGGGGTCGGTTCACAGGTGGTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCAC
TGTTGCCAGCACGTTATGGTGGGGACTCAGTGGAGACCGCCGGGGTCAACTCGGAGGAAGGT
GGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACGCATGCTACAATGGCTGG
TACAGAGAGTGGCGAGCCTGTGAGGGTGAGCGAATCTCGGAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCTCATGA

6.2. Non cultured bacteria

6.2.1. *Gemella sp*

Range 459/476 Identity 96% Sample 15 A1

AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGA
AGTTTAAGAGATGCTTGCATCACTTGAACTTAGCGGCGAACGGGTGAGTAACACGTAAAGAA
CCTGCCTCATAGACTGGGACAACTACTGGAAACGGTAGCTAATACCGGATAATATATTTCTTTG
CATGAAGGAATATTGAAAGTCGGTCAAGCTGACACTATGAGATGGCTTTGCGGTGCATTAGCT
AGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTTGTAAAAC
TCTGTTGTTAGGGAAGAATGATTGTATAGTAACTATATACAGTAGCGACGGTACCTAC

6.2.2. *Lactobacillus sp*

Range 484/486 Identity 99% Sample 15 G9

AGAGTTTGATTATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGA
AACTTCTTTATCACCGAGTGCTTGCACTCACCGATAAAGAGTTGAGTGGCGAACGGGTGAGTA
ACACGTGGGCAACCTGCCCAAAAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCAT
AACCATAGTTACCGCATGGTAACTATGTAAAAGGTGGCTATGCTACCGCTTTTGGATGGGCCC
GCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCTTACCAAGGCAATGATGCGTAGCCGAACTG
AGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGGGTGAAGAAGGTCTT
CGGATCGTAAAACCCTGTTGTTAGAGAAGAAAGTGCGTGAGAGTAACTGTTCACGTTCGACG
GTATCTAC

6.2.3. *Streptococcus sp*

Range 456/477 Identity 96% Sample 15 D4

AGAGTTTGATTATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCT
GAAGAAAGAGCTTGCTCTTTTGGAAGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGC
CTGGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAATAGCTTTGATTGCATG
ATTGAAGTTTGAAAGATGCAACTGCATCACTACCAGATGGACCTGCGTTGTATTAGCTAGTAG
GTGAGGTAAAGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGAACGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGG
GGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGT
TGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTACTACA

6.2.4. *Cellulomonas sp*

Range 444/465 Identity 95% Sample 15 E6

AGAGTTTGATTCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGG
TGAACCCCAGCTTGCTGGGGGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCC
CCTGACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACCGGATATGAGGCGCTCGGGCATC
CGAAGCGTCTGGAAAGATTTATCGGTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGG
GGTAGTGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGC
AGGGAAGAAGCGAGAGTGACGGTACTGCA

6.2.5. *Staphylococcus sp*

Range 484/492 Identity 98% Sample 15 A5

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGA
ACAGATGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACCGC
ATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCT
AGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTAGGATCGTAAAACT
CTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTGAACAAGTCTGACGGTACCTAC

6.2.6. *Clostridium sp*

Range 416/462 Identity 90% Sample 15 G4

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCCTAATACATGCAAGTCGAACGA
AGACTGTTGAAAGCTTGCTTTTGACAGACTTAGTGGCGAACGGGTGAGTAACACGTAGGGAA
CCTGCCCATGTACCCGGGATAACAGATGGAAACGTCTGCTAAAACCGGATAGGTATAGACAA
GGCATCTTGACTATATTAAAGGACCTTCGGGTGCGGACATGGATGGACCTGCGGCGTATTAGC
CAGTTGGCGGGGTAACGGCCCACCAAAGCGATGATACGTAGCCGGCCTGAGAGGGTAGACG
GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGT
CAATGGGGGAAACCCTGAACGAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAA
GCTCTGTTGTAAGAGAAAAACGGTAATGATAGGGAATGATCATTAAGTGATGGTTC

6.2.7. *Acinetobacter sp*

Range 466/473 Identity 99% Sample 15 A2

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGG
GGAAAGGTAGCTTGCTACCTGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCT
ATTAGTGGGGGACAACATTCCGAAAGGAATGCTAATACCGCATACGCCCTACGGGGGAAAGC
AGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCAGATTAGCTAGTTGGTGGGGTA
AAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC
CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGA
GGAGGCTACTTAGATTAATACTCTAGGATAGTGGACGTACTCGCAGAT

6.2.8. *Agrobacterium sp*

Range 395/397 Identity 99% Sample 15 H4

AGAGTTTGATTATGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGC
CCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGCGTGGGAACATACCCTTTCCTGCGGAATA
GCTCCGGGAAACTGGAATTAATACCGCATACGCCCTACGGGGGAAAGATTTATCGGGGAAGG
ATTGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAG
CTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATG
AAGGCCTTAGGGTTGTAAAGCTCTTCACCGGAGAAGATAATGACGGTTCC

6.2.9. *Microbacterium sp*

Range 442/444 Identity 99% Sample 5 E4

AGAGTTTGATTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGT
GAAGCAGAGCTTGCTCTGTGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAATCTGCCC
CTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACCGGATACGAGCTGCGACCGCATGG
TCAGTAGCTGGAAAGCATTTCGGTCAGGGATGAGCTCGCGGCCTATCGGCTTGTTGGTGAGG
TAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGG
GAAGAAGCGAAAGTGACGGTACCTGCAGA

6.2.10. *Exiguobacterium sp*

Range 503/508 Identity 99% Sample 5 D4

AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGC
AGGAAGCTCACGGAACTCTTCGGAGGGAAGTGAAGGGAATGAGCGGCGGACGGGTGAGTAA
CACGTAAGGAACCTGCCCCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGAGTA
GTTCTTCAGACCGCATGGTCTGATGATGAAAGGCGCTCCGGCGTCACCTTGGGATGGCCTTGC
GGTGCATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGACGATGCATAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGAGAGGGAATGCTCGTACCTTGACG
GTACCTGCGAGA

6.2.11. *Acidovorax sp*

Range 461/480 Identity 96% Sample 5 A9

AGAGTTTGATTATGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGT
AGCAGCTCTTCGGAGGCTGACGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCGAT
CGTGGGGGATAACGGAGCGAAAGCTTTGCTAATACCGCATACGATCTACGGATGAAAGCAGG
GGACCCTCGGGCCTTGCGCGAACGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAA
AAGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCT
GATCCAGCAATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACG
AAAAGTCTCGGGATAATACCCTGGGGCCATGACGGTACCGTAAGAA

6.2.12. *Sanguibacter sp*

Range 457/461 Identity 99% Sample 6 E10

AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGG
TGACGCTAGAGCTTGCTCTGGTTGATCAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTG
CCCTTGACTCTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATACGAGACGCGACCGCA
TGGTCGGCGTCTGGAAAGTTTTTCGGTCAAGGATGGACTCGCGGCCTATCAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGTCGACGGGTAGCCGACCTGAGAGGGCGACCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
AAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGT
AGGGAAGAAGCGAAAGTGACGGTACTGCAG

6.2.13. *Pseudomonas sp*

Range 477/483 Identity 99% Sample 6 D12

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
TAGAGAGGAGCTTGCTCCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCT
GGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGC
AGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG
CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGG
AGGAAGGGCAGTAAATTAATACTTTGCTGTTTGACGTACCGACAGAATAG

6.2.14. *Janthinobacterium sp*

Range 456/460 Identity 99% Sample 6 A10

AGAGTTTGATTATGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGC
AGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGTACCCT
GGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTACGGATGAAAGTG
GGGGATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTA
AAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGC
CTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGA
AGAAACGGTGAGGGCTAATATCCCTTGCTAATGACGGTACCTAAG

6.2.15. *Enterobacter sp*

Range481/489 Identity 99% Sample 6 A2

AGAGTTTGATTATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
TAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGGGTAATGTCTGGGAAACT
GCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCA
AAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTG
GGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG
AGGAGGAAGGCATTGTGGTTAATAACCGCAGTGATGACGTACTCGCAGAAGAG

6.2.16. *Psychrobacter sp*

Range 481/487 Identity 99% Sample 6 A7

AGAGTTTGATTATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGG
AAACGATGATAGCTTGCTATCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCT
ACCTAGTAGTGGGGGATAGCACGGGGAAACTCGTATTAATACCGCATACGACCTACGGGAGA
AAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGG
GTAAAGGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACCGGGA
CTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAA
ACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCAG
TGAAGAAGACTCCGTGGTTAATACCCACGGACGATGACATAGCTGCAGAATA

6.2.17. *Comamonas sp*

Range 442/452 Identity 98% Sample 6 A4

GAGTTTGATTCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGTA
ACAGGTCTTCGGACGCTGACGAGTGGCGAACGGGTGAGTAACACATCGGAACGTGCCTAGTA
GTGGGGGATAACTACTCGAAAGAGTAGCTAATACCGCATGAGATCTAAGGATGAAAGCAGGG
GACCTTCGGGCCTTGCGCTACTAGAGCGGCTGATGGCAGATTAGGTAGTTGGTGGGATAAAA
GCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAACAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGA
TCCAGCAATGCCGCGTGCAGGATGAAGGCCCTCGGGTTGTAAACTGCTTTTGTACGGAACGAA
AAGACTCTCTCTAATAAAGAGGGTTGATGACGGTACCGTAAGAT

6.2.18. *Massilia sp*

Range 462/465 Identity 99% Sample 6 D7

AGAGTTTGATTCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGC
AGCGCGGGGCAACCTGGCGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCAA
GAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGG
GGGATCGCAAGACCTCATGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTGAGGTAAA
GGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTG
ATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAG
AAACGGTAGGAGCTAATATCTCTTGCTAATGACGGTACCTAAG

6.2.19. *Helicobacter sp*

Range 424/424 Identity 100% Sample 7 H8

AGAGTTTGATTATGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAT
GAAGCTCTAGCTTGCTAGAGTGGATTAGTGGCGCACGGGTGAGTAATGCATAGATAACATGC
CCTTTAGTCTAGGATAGCCATTGGAAACGATGATTAATACTGGATACTCCTTACGAGGGAAAG
TTTTTCGCTAAAGGATTGGTCTATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGC
TATGACGGGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAACCCTGAAGCAGCAACGCC
GCGTGGAGGATGAAGGTTTTCGGATTGTAAACTCCTTTTGTTAGAGAAGATAATGACGGTATC
TAACG

6.2.20. *Bacteroides sp*

Range 440/451 Identity 98% Sample 8 A10

AGAGTTTGATTATGGCTCAGGATGAACGCTAGCCACAGGCTTAACACATGCAAGTCGAGGGG
CAGCATGAACTTAGCTTGCTAAGTTTGATGGCGACCGGCGCACGGGTGAGTAACACGTATCCA
ACCTGCCGATGACTCGGGGATAGCCTTTCGAAAGAAAGATTAATACCCGATGGCATAGTTCTT
CCGCATGGTAGAACTATTAAAGAATTTCGGTCATCGATGGGGATGCGTTCCATTAGCTTGTTG
GCGGGGTAACGGCCCACCAAGGCATCGATGGATAGGGGTTCTGAGAGGAAGGTCCCCCACAT
TGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGG
CGCTAGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAAACTTCTTTT
ATATGGGAATAAAGTGCAGTATGTATACTGCTT-GCATGTACCTAG

6.2.21. *Abiotrophia sp*

Range 487/494 Identity 99% Sample 12 C4

AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGA
ACCGCGACTAGGTGCTTGCACTTGGTCAAGGTGAGTGGCGAACGGGTGAGTAACACGTGGGT
AACCTACCTCATAGTGGGGGATAACAGTCGGAAACGACTGCTAATACCGCATAGGACGTGGG
ATTACATGATTCTGTGAGGAAAGGTGGCGCAAGCTATCGCTAAGAGATGGACCCGCGGTGCA
TTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGACCTGAGAGGGTG
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGATCGT
AAAGCTCTGTTGTTAGAGAAGAACAGCGCATAGAGTAACTGTTATGCGTGTGACGGTATC

6.2.22. *Kocuria sp*

Range 459/470 Identity 98% Sample 12 C12

AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGCT
GAAGCTTGGTGCTTGCACTGGGTGGATGAGTGGCGAACGGGTGAGTAATACGTGAGTAACCT
GCCCTTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACATGTCACCGCA
TGGTGGTGTGTGGAAAGGGTTTTACTGGTTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGT
GGGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTC
AGCACGGAAGAAGCGAAAGTGACGGTACGGCAG

6.2.23. *Geobacillus sp*

Range 471/474 Identity 99% Sample 12 G7

AGAGTTTGATTCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGG
ACCGAAGGGAGCTTGCTCCTTTAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTG
CCCTGCAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCA
TGGTTTTCGGTTGAAAGGCGGCTTTTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCCGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAG
CTCTGTTGTCAGGGAAGAACAAGTGCCGTTCGAACAGGGCGGTACCTGACGGTAC

6.2.24. *Duganella sp*

Range 421/421 Identity 100% Sample 13 A12

AGAGTTTGATTATGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGC
AGCGCGGGGCAACCTGGCGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTG
GAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGG
GGGATCGCAAGACCTCATGCTCCTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAA
AGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTG
ATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAG
AAACGGTG

6.2.25. *Paracoccus sp*

Range 408/411 Identity 99% Sample 12 E5

AGAGTTTGATTCTGGCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGC
CCTTTCGGGGGAGCGGCGGACGGGTGAGTAACGCGTGGGAATATGCCCTTCTCTACGGAATA
GCCTCGGGAAACTGAGAGTAATACCGTATACGCCCTGTGGGGGAAAGATTTATCGGGGAAGG
ATTAGCCCGCGTTGGATTAGGTAGTTGGTGGGGTAATGGCCTACCAAGCCGACGATCCATAGC
TGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATCTTAGACAATGGGGGCAACCCTGATCTAGCCATGCCGCGTGAGTGATGA
AGGCCTTAGGGTTGTAAAGCTCTTTCAGCTGGGAAGATAATGACGTAC

6.2.26. *Chryseobacterium sp*

Range 455/477 Identity 95% Sample 14 C10

AGAGTTTGATTATGGCTCAGGATGAACGCTAGCGGGAGGCCTAACACATGCAAGCCGAGCGG
TATTTGTTCTTCGGAACAGAGAGAGCGGCGTACGGGTGCGTAACACGTGTGCAACCTACCTTT
ATCAGGGGAATAGCCTTTCGAAAGGAAGATTAATACTCCATAATATATTTCATGGCATCATTGG
ATATTGAAAACTCCGGTGGATAGAGATGGGCACGCGCAAGATTAGATAGTTGGTGAGGTAAC
GGCTCACCAAGTCTGTGATCTTTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGA
CACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGTGAGAGCCTG
ATCCAGCCATCCCGCGTGAAGGATTAAGGTCCTACGGATTGTAAACTTCTTTTGTACAGGAATA
AACCTATTTACGTGTAGATAGCTGAAGGTACTTACG

6.2.27. *Stenotrophomonas sp*

Range 474/480 Identity 99% Sample 14 B6

AGAGTTTGATTATGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGG
CAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAA
TCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGT
GAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGG
CGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG
CGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTG
TTGGGAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGTAC

6.2.28. *Carnobacterium sp*

Range

AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCATGCCTAATACATGCAAGTCGAACGCT
TTTGTTTCACCGGGTGCTTGCACCCACCGAGACAAAAGAGTGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCCATAAGAGGGGGATAACATCCGGAAACGGATGCTAATACCGCATATTTCC
AATTGTCTCCTGACAGATGGAAAAAAGGTGGCTTCGGCTACCGCTTATGGATGGACCCGCGGC
GTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGA
TCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGCTCATCCCCTGACGGTAT
CTA

6.2.29. *Roseburia sp*

Range 441/487 Identity 91% Sample 11 E4

AGAGTTTGATTATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGA
AGCACCTGAGAATGATTCTTCGGATGAAGTCTCTTGTGACTGAGTGGCGGACGGGTGAGTAA
CGCGTGGGTAACCTGCCTCACACAGGGGGATAACAGCTGGAAACGACCGCTAATACCGCATA
ACCCGCTGGTGCCGCATGGCACTGACGGAAAAGATTTATCGGTGTGAGGTGGACCCGCGTCT
GATTAGCCAGTTGGCAGGGTAACGGCCTACCAAAGCGACGATCAGTAGCCGGCCTGAGAGGG
TGAACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTGCGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGTGAAGAAGTATTTCGGTA
TGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTGATAA

6.2.30. *Delftia sp*

Range 477/479 Identity 99% Sample 11 G7

AGAGTTTGATTCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGT
AACAGGTCTTCGGACGCTGACGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCAGT
CGTGGGGGGTAACTACTCGAAAGAGTAGCTAATACCGCATACGATCTGAGGATGAAAGCGGG
GGACCTTCGGGCCTCGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAA
AGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGA
AAAAGCTTCTCCTAATACGAGAGGCCCATGACGGTACCGTAAG

6.2.31. *Ochrobactrum sp*

Range 409/433 Identity 94% Sample 11 D2

AGAGTTTGATTATGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGG
TCTCTTCGGAGGCAGTGGCAGACGGGTGAGTAACGCGTGGGAATCTACCTTTTGCTACGGAAC
AACAGTTGGAAACGACTGCTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGGCAAAG
GATGAGCCCGCGTTGGATTAGCTAGTTGGTAGGGTAAGGGCCTACCAAGGCGACGATCCATA
GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT
GAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGAGAGA

6.2.32. *Flavobacterium sp*

Range 456/476 Identity 96% Sample 11 D7

AGAGTTTGATTATGGCTCAGGATGAACGCTAGCGGCGGGCTTAACACATGCAAGTCGAGGGG
TATACTTCTTCGGAAGTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTACCTTTTA
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GCAATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAGCTAGTTGGTAAGGTAACG
GCTTACCAAGGCTACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGA
CACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTATACAGGAAG
AAACCCTGGTTCGTGAACCAGCTGACGGTACGTAAGA

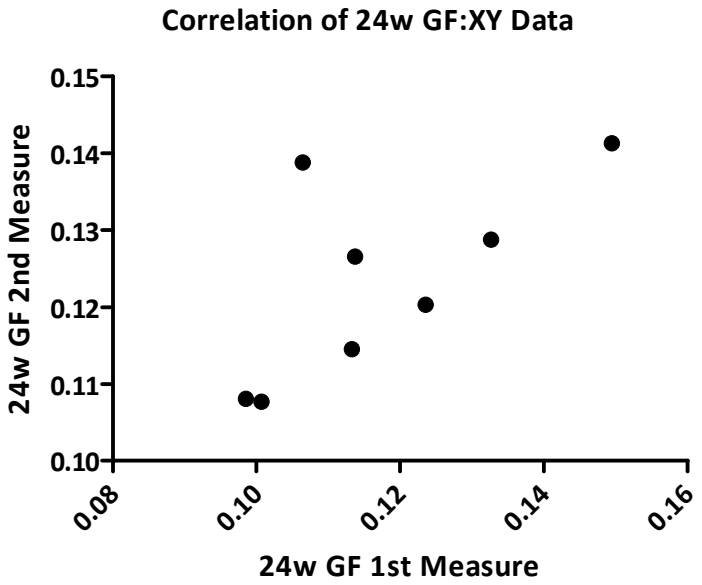
7. Bone loss – Intra-operator reliability

7.1. Aging C3H/Orl germ free (GF) and Specific Pathogen Free (SPF) mice (see Figure 4.10)

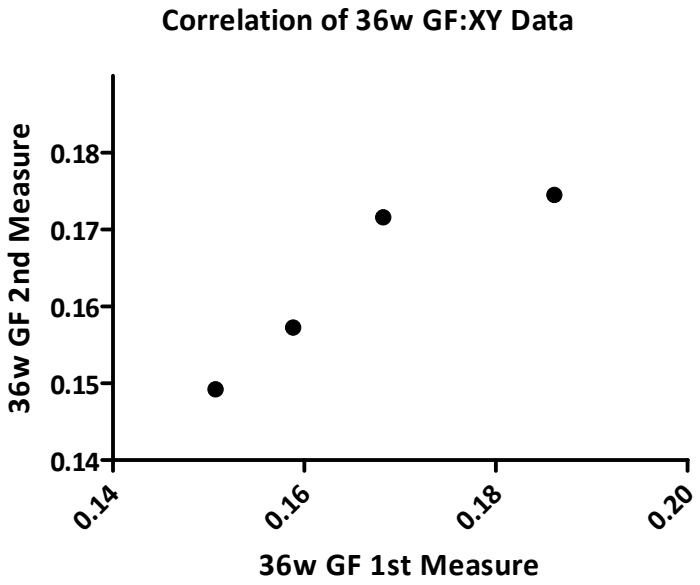
Germ Free (GF) Intra-operator reliability

10w GF 1st Measure	10w GF 2nd Measure	24w GF 1st Measure	24w GF 2nd Measure	36w GF 1st Measure	36w GF 2nd Measure	78w GF 1st Measure	78w GF 2nd Measure
0.136520	0.135826	0.113732	0.126570	0.186072	0.174537	0.349559	0.381569
0.140373	0.117921	0.113263	0.114541	0.168231	0.171598	0.396118	0.392573
		0.149427	0.141324	0.158832	0.157258	0.408712	0.414712
		0.106419	0.138792	0.150723	0.149237	0.420496	0.431051
		0.100674	0.107681			0.431279	0.430526
		0.098518	0.108057				
		0.132665	0.128765				
		0.123543	0.120306				
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
Not possible as only 2 pairs of values		r=0.6703 p=0.0689 95% CI -0.0654-0.9339		r=0.9209 p=0.0791 95% CI -0.3499-0.9984		r=0.9221 p=0.0258 95% CI 0.2134-0.9949	

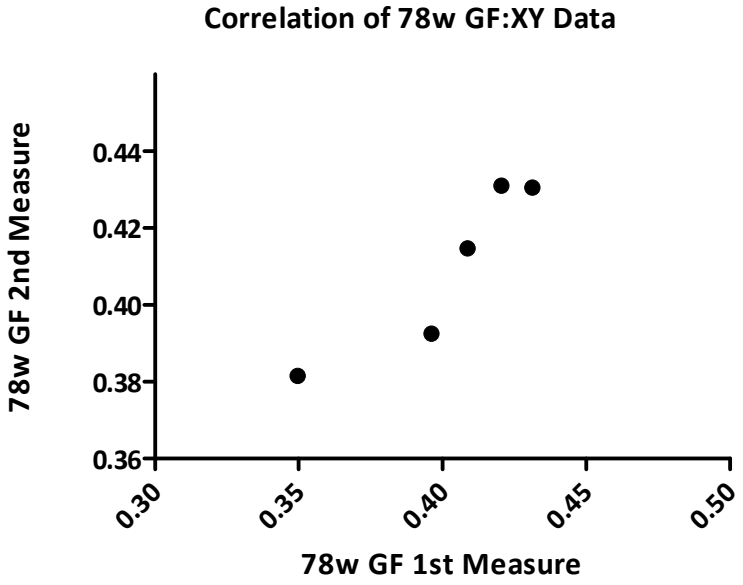
24 week Germ Free (GF) bone loss data



36 week Germ Free (GF) bone loss data



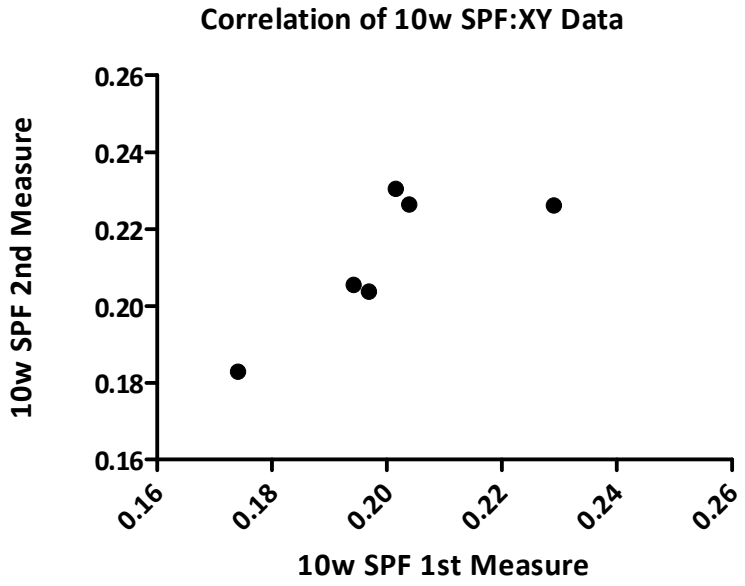
78 week Germ Free (GF) bone loss data



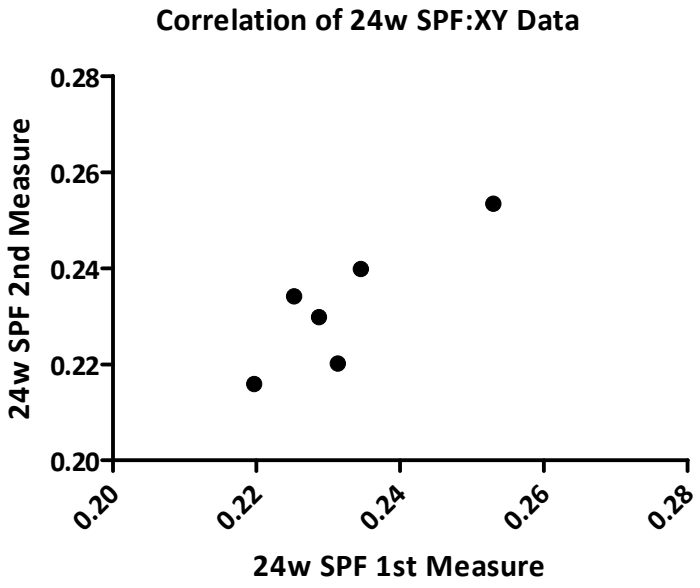
Specific Pathogen Free (SPF) Intra-operator reliability

10w SPF 1st Measure	10w SPF 2nd Measure	24w SPF 1st Measure	24w SPF 2nd Measure	36w SPF 1st Measure	36w SPF 2nd Measure	78w SPF 1st Measure	78w SPF 2nd Measure
0.229011	0.226094	0.219685	0.215902	0.251566	0.254468	0.338043	0.325738
0.201551	0.230432	0.228674	0.229866	0.265967	0.254311	0.325158	0.322065
0.203868	0.226339	0.225226	0.234174	0.275297	0.283681	0.299350	0.271521
0.174111	0.182851	0.231303	0.220140	0.271873	0.266389	0.323442	0.326729
0.194186	0.205472	0.234499	0.239818	0.272957	0.285259		
0.196867	0.203709	0.252976	0.253458	0.273508	0.280458		
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.8013 p=0.0553 95% CI -0.0296-0.9973		r=0.8561 p=0.0296 95% CI 0.1456-0.9840		r=0.7906 p=0.0612 95% CI -0.0588-0.9760		r=0.9187 p=0.0813 95% CI -0.3626-0.9983	

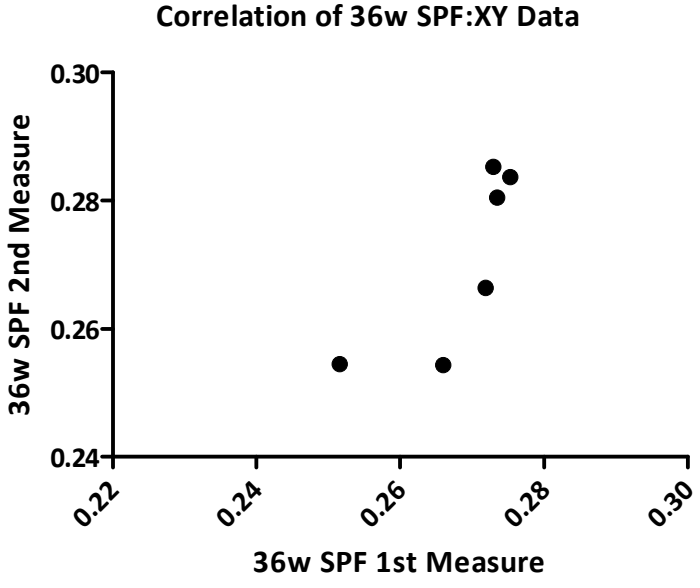
10 week Specific Pathogen Free (SPF) bone loss data



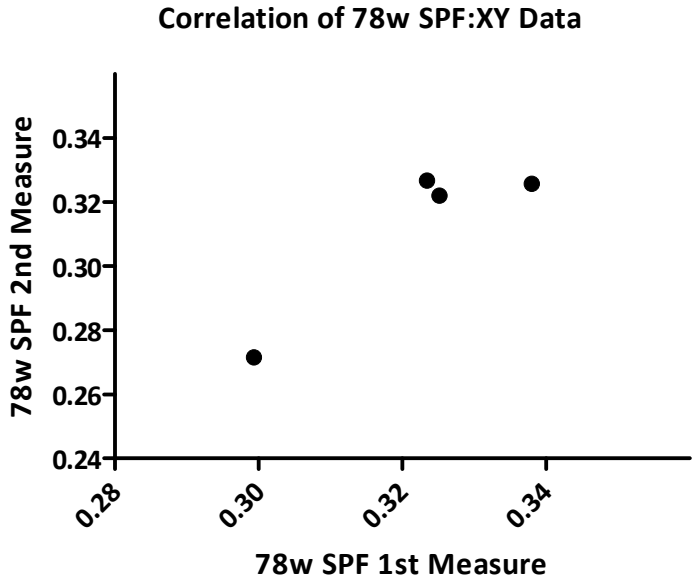
24 week Specific Pathogen Free (SPF) bone loss data



36 week Specific Pathogen Free (SPF) bone loss data



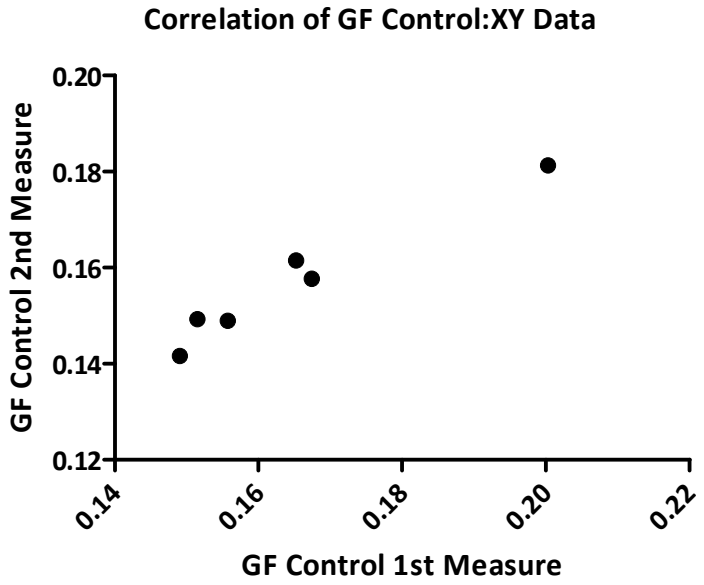
78 week Specific Pathogen Free (SPF) bone loss data



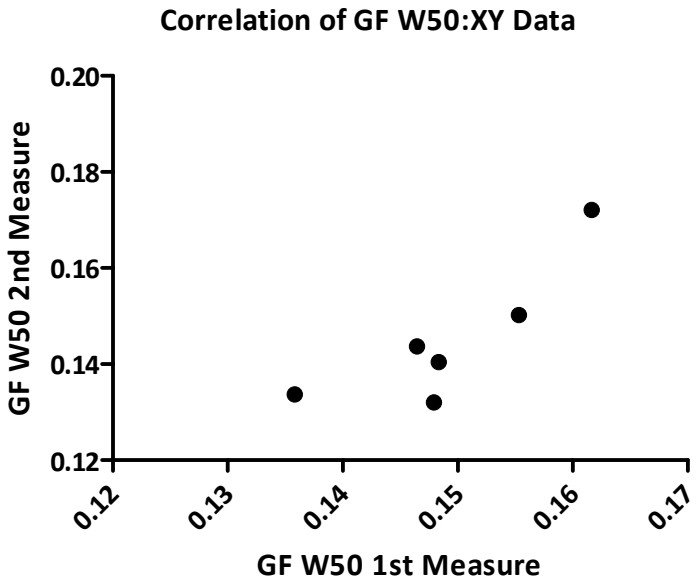
7.2. *Porphyromonas gingivalis* challenge of Specific Pathogen Free (SPF) and Germ Free (GF) mice (See figure 4.14)

GF Control 1st Measure	GF Control 2nd Measure	GF W50 1st Measure	GF W50 2nd Measure	SPF Control 1st Measure	SPF Control 2nd Measure	SPF W50 1st Measure	SPF W50 2nd Measure
0.155712	0.148962	0.146455	0.143716	0.304394	0.297346	0.496831	0.528305
0.200289	0.181281	0.161639	0.172094	0.388167	0.383763	0.595306	0.562683
0.149056	0.141661	0.155319	0.150250	0.409431	0.422056	0.492139	0.510591
0.151500	0.149270	0.135833	0.133709	0.455653	0.506645	0.492889	0.492675
0.167389	0.157722	0.148361	0.140409	0.399139	0.461554	0.489556	0.487963
0.165236	0.161517	0.147939	0.132042			0.481917	0.468323
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.9783 p=0.0007 95% CI 0.8090-0.9977		r=0.8483 p=0.0328 95% CI 0.1177-0.9831		r=0.9530 p=0.0121 95% CI 0.4441-0.9970		r=0.8521 p=0.0312 95% CI 0.1311-0.9835	

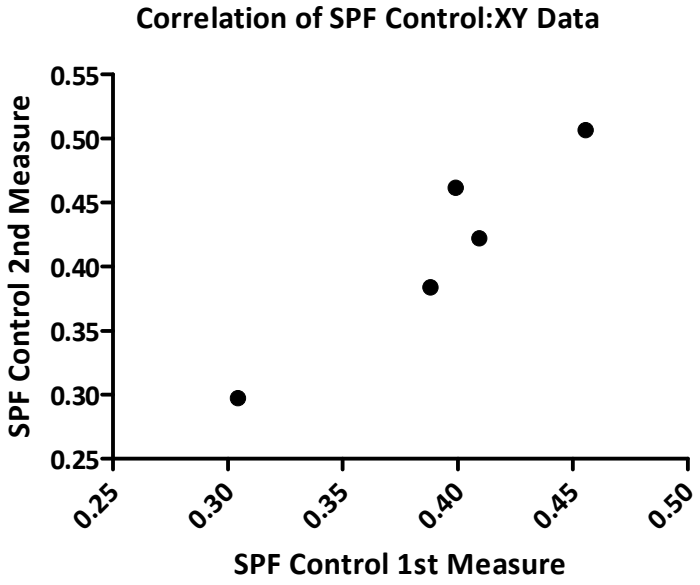
Germ Free (GF) Control bone loss data



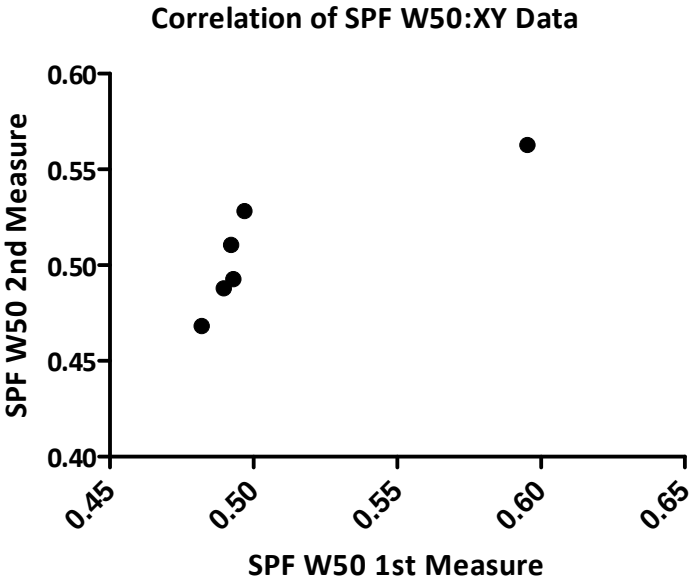
Germ Free W50 (GFW50)



Specific Pathogen Free (SPF) Control bone loss data



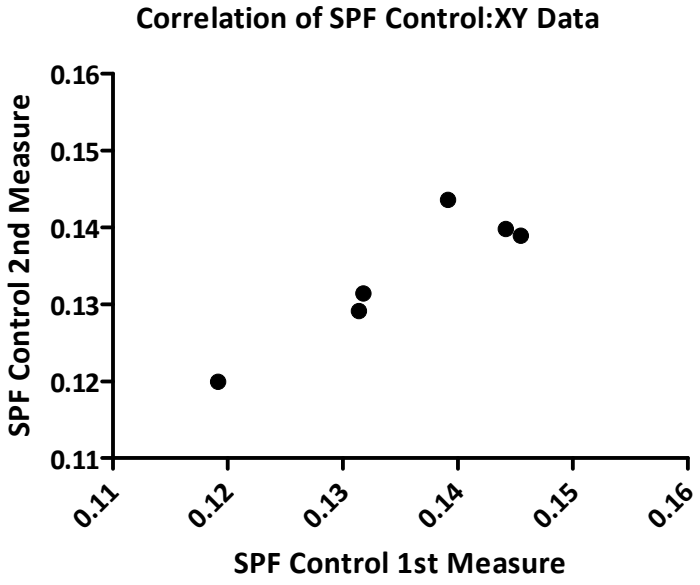
Specific Pathogen Free W50 (SPFW50) bone loss data



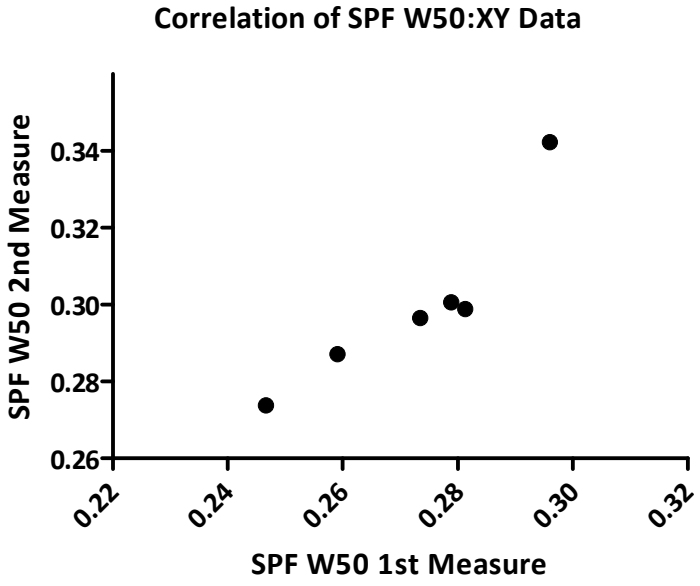
7.3. *Porphyromonas gingivalis* challenge of Specfific Pathogen Free (SPF) and Germ Free (GF) mice (See figure 4.18)

SPF Control 1st Measure	SPF Control 2nd Measure	SPF W50 1st Measure	SPF W50 2nd Measure
0.144193	0.139798	0.259107	0.287148
0.131792	0.131434	0.296039	0.342277
0.131397	0.129153	0.278859	0.300623
0.139166	0.143614	0.281290	0.298897
0.119161	0.119957	0.246615	0.273790
0.145490	0.138943	0.273480	0.296541
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**	
r=0.9189 p=0.0096 95% CI 0.4219-0.9913		r=0.9140 p=0.0108 95% CI 0.3963-0.9907	

Specific Pathogen Free (SPF) Control bone loss data



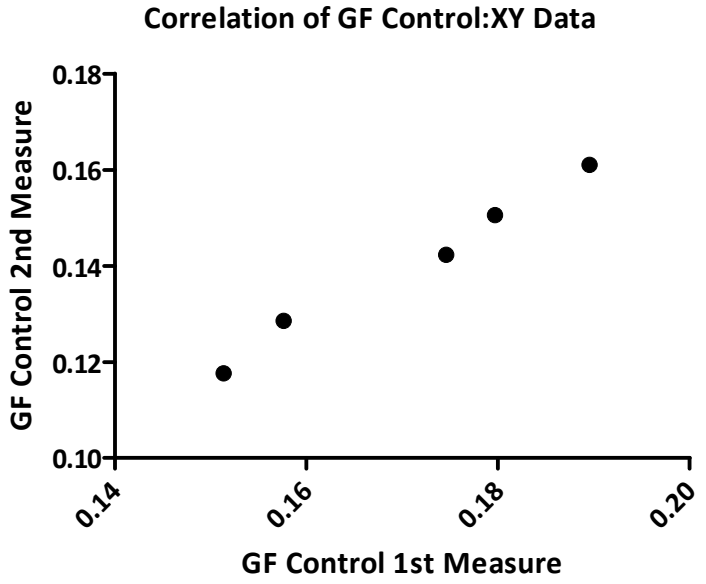
Specific Pathogen Free W50 (SPF W50) bone loss data



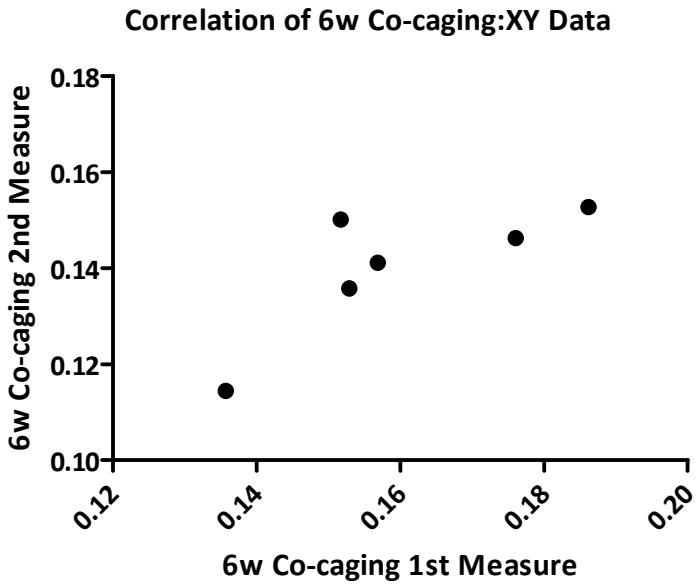
7.4. Transmission of commensal microbiota between Specific Pathogen Free (SPF) and Germ Free (GF) C3H/Orl mice (See Figure 4.34)

GF Control 1st Measure	GF Control 2nd Measure	6w Co-caging 1st Measure	6w Co-caging 2nd Measure	16w Co-caging 1st Measure	16w Co-caging 2nd Measure	SPF Control 1st Measure	SPF Control 2nd Measure
0.151357	0.117643	0.151704	0.150068	0.420106	0.362589	0.304394	0.297346
0.157611	0.128563	0.156847	0.141133	0.448556	0.407432	0.388167	0.383763
0.179667	0.150542	0.135722	0.114426	0.373000	0.345581	0.409431	0.422056
0.174576	0.142313	0.152909	0.135761	0.359278	0.308542	0.455653	0.506645
0.189542	0.161029	0.186181	0.152713			0.399139	0.461554
		0.176083	0.146261				
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.9941 p=0.0005 95% CI 0.9099-0.9996		r=0.7832 p=0.0654 95% CI -0.0708-0.9750		r=0.9499 p=0.0501 95% CI -0.1287-0.9990		r=0.9530 p=0.0121 95% CI 0.4441-0.9970	

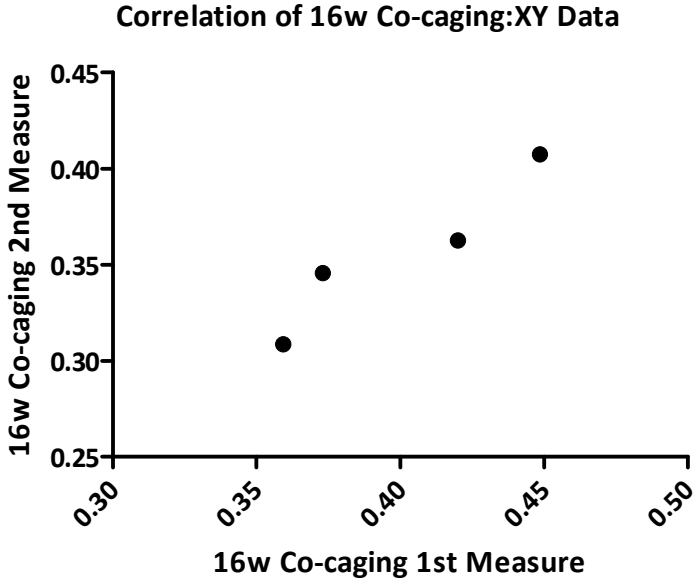
Germ Free (GF) Control bone loss data



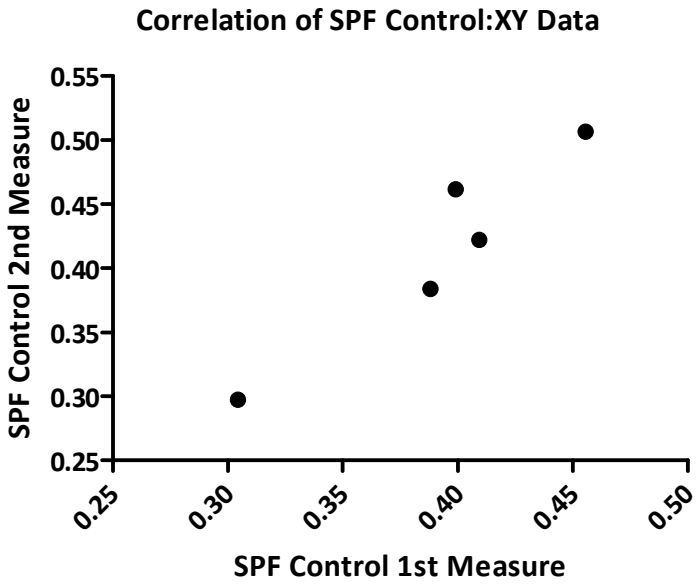
6 week Co-caging bone loss data



16 week Co-caging bone loss data



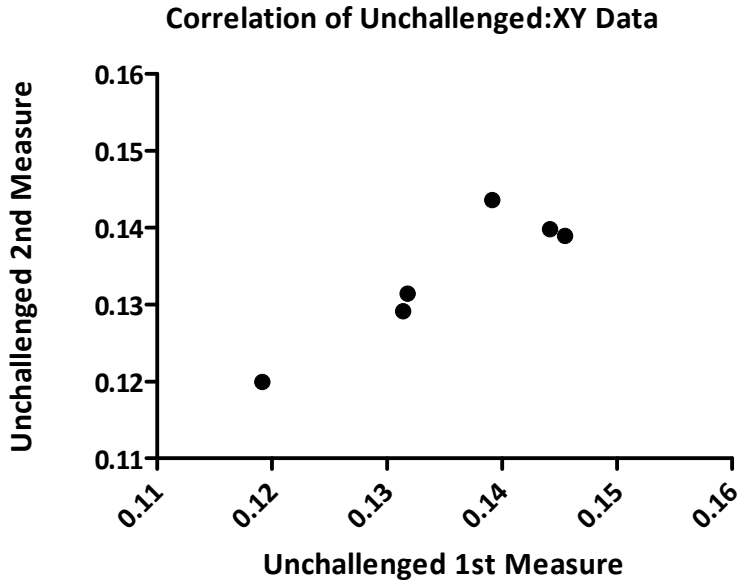
Specific Pathogen Free (SPF) Control



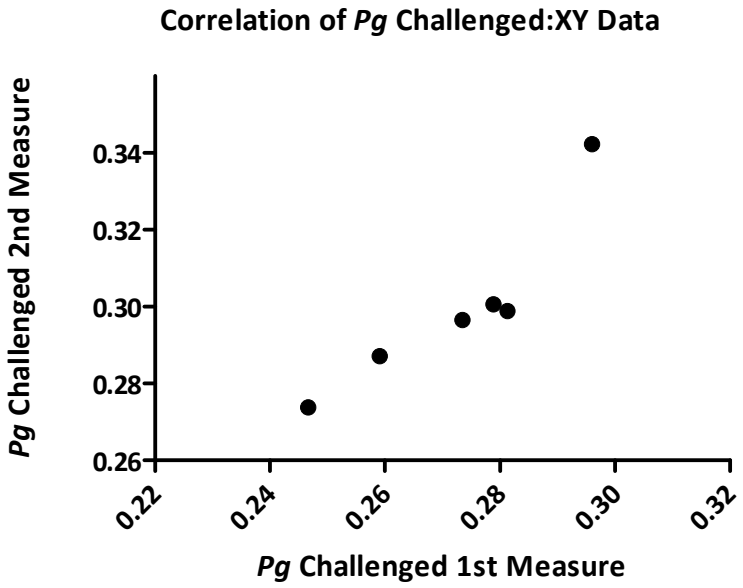
7.5. Transmission of *P. gingivalis* altered commensal microbiota from SPF into GF mice (See Figure 4.39)

Un-challenged 1st Measure	Un-challenged 2nd Measure	*Pg* Challenged 1st Measure	*Pg* Challenged 2nd Measure	Conventionalised 1st Measure	Conventionalised 2nd Measure
0.144193	0.139798	0.259107	0.287148	0.260431	0.273644
0.131792	0.131434	0.296039	0.342277	0.248722	0.233436
0.131397	0.129153	0.278859	0.300623	0.289461	0.278421
0.139166	0.143614	0.281290	0.298897	0.270823	0.258471
0.119161	0.119957	0.246615	0.273790		
0.145490	0.138943	0.273480	0.296541		
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.9189 p=0.0096 95% CI 0.4219-0.9913		r=0.9140 p=0.0108 95% CI 0.3963-0.9907		r=0.9306 p=0.0694 95% CI -0.2892-0.9986	

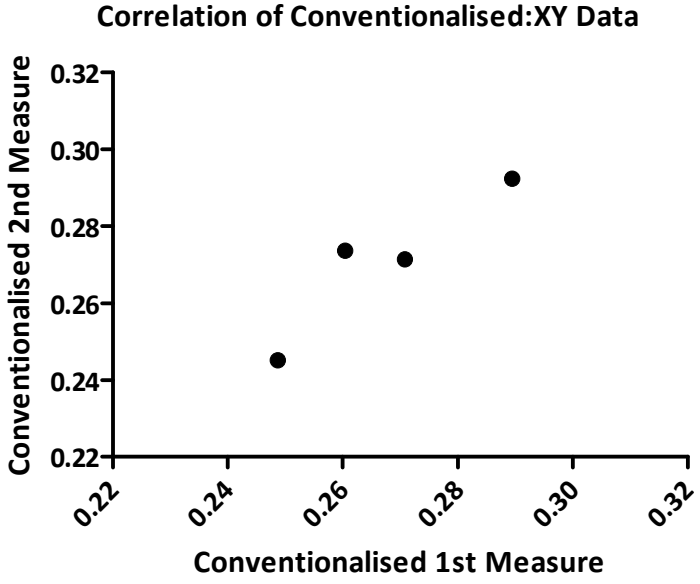
Unchallenged bone loss data



***Pg* challenged bone loss data**



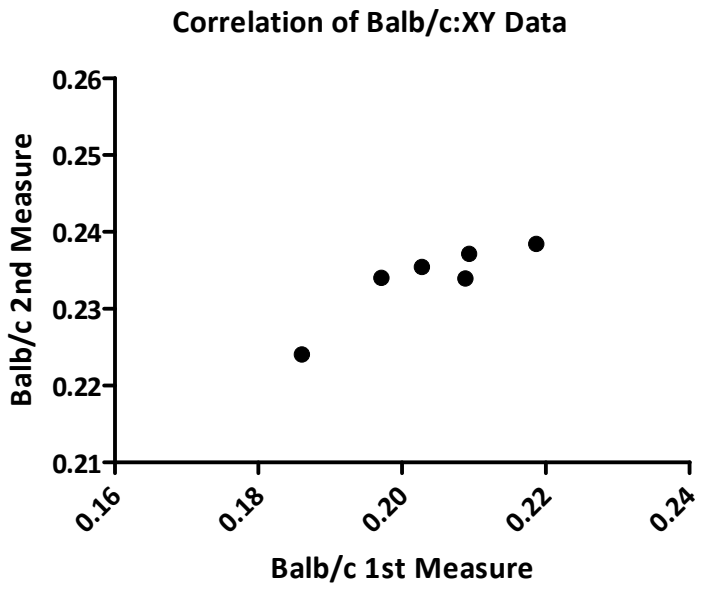
Conventionalised bone loss data



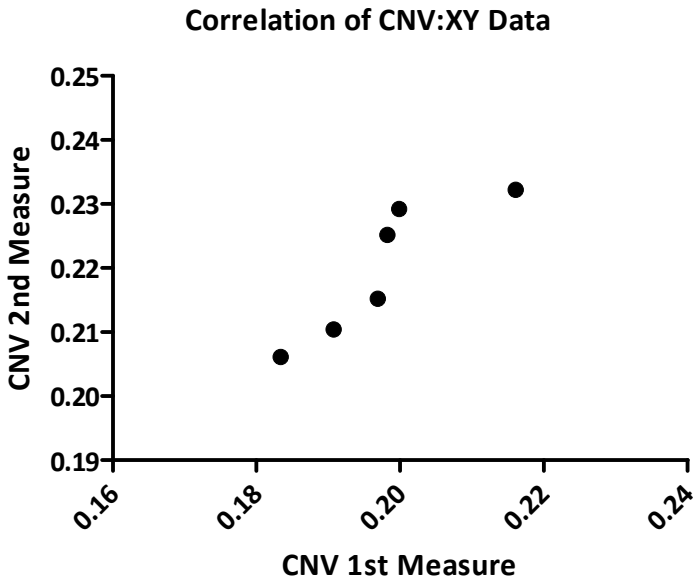
7.6. Transmission of commensal microbiota from SPF Balb/cAnNCrl mouse to C3H/Orl GF mouse (See Figure 4.50)

Balb/c 1st Measure	Balb/c 2nd Measure	CNV 1st Measure	CNV 2nd Measure	SPF 1st Measure	SPF 2nd Measure
0.202742	0.235430	0.199839	0.229245	0.219685	0.226823
0.197067	0.234022	0.183345	0.206114	0.228674	0.223389
0.186003	0.224080	0.216056	0.232220	0.225226	0.220513
0.209306	0.237139	0.198193	0.225157	0.231303	0.233198
0.218647	0.238429	0.196870	0.215225	0.234499	0.244845
0.208781	0.233957	0.190754	0.210420	0.252976	0.252574
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.8950 p=0.0160 95% CI 0.3045-0.9885		r=-0.8864 p=0.0186 95% CI 0.2666-0.9876		r=0.8691 p=0.0246 95% CI 0.1951-0.9855	

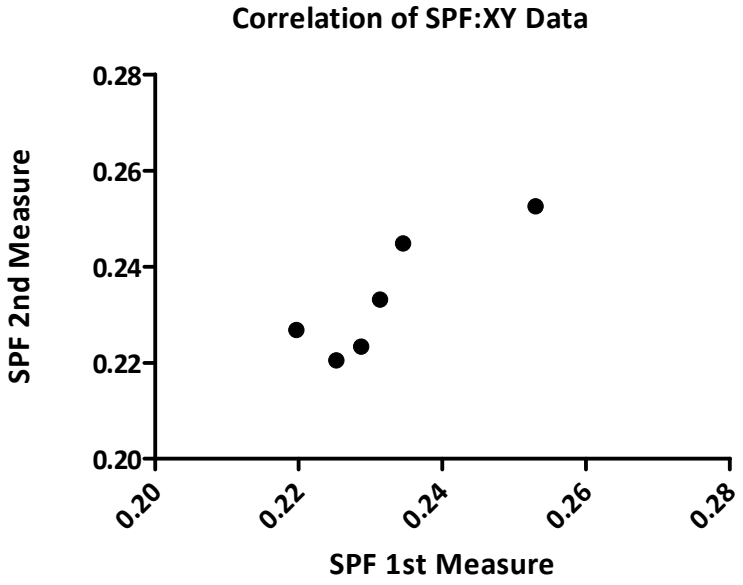
Balb/c bone loss data



CNV bone loss data



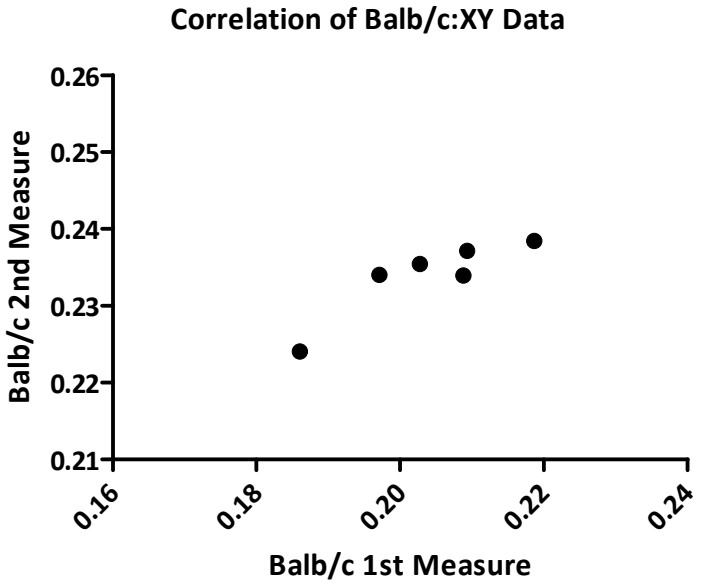
SPF bone loss data



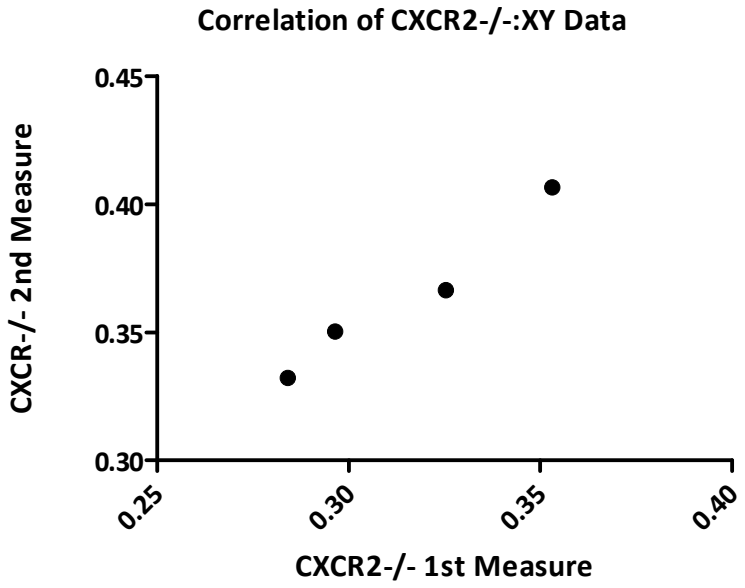
7.7. SPF CXCR2$^{-/-}$ knockout mice (See Figure 4.55)

Balb/c 1st Measure	Balb/c 2nd Measure	CXCR2$^{-/-}$ 1st Measure	CXCR2$^{-/-}$ 2nd Measure
0.202742	0.235430	0.296449	0.350359
0.197067	0.234022	0.325410	0.366440
0.186003	0.224080	0.284154	0.332208
0.209306	0.237139	0.353137	0.406564
0.218647	0.238429		
0.208781	0.233957		
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**	
r=0.8950 p=0.0160 95% CI 0.3045-0.9885		r=-0.9819 p=0.0181 95% CI 0.3703-0.9996	

Balb/c bone loss data



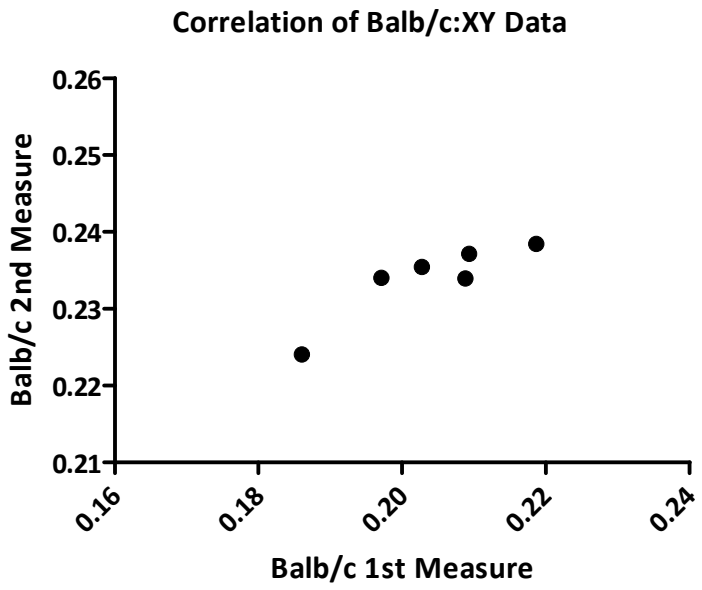
CXCR2$^{-/-}$ bone loss data



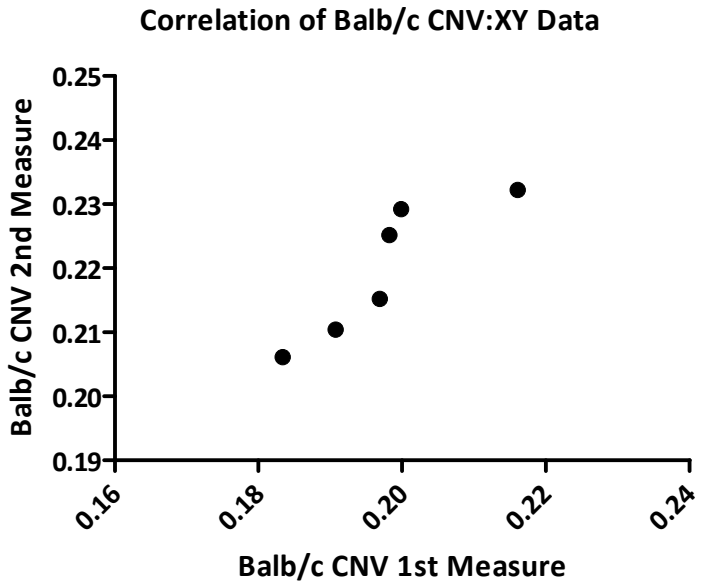
7.8. Transmission of commensal microbiota from SPF CXCR2-/- mouse to GF C3H/Orl mouse (See Figure 4.61)

Balb/c 1st Measure	Balb/c 2nd Measure	Balb/c CNV 1st Measure	Balb/c CNV 2nd Measure	$CXCR2^{-/-}$ 1st Measure	$CXCR2^{-/-}$ 2nd Measure	$CXCR2^{-/-}$ CNV 1st Measure	$CXCR2^{-/-}$ CNV 2nd Measure
0.202742	0.235430	0.199839	0.229245	0.296449	0.350359	0.117218	0.143159
0.197067	0.234022	0.183345	0.206114	0.325410	0.366440	0.121152	0.120191
0.186003	0.224080	0.216056	0.232220	0.284154	0.332208	0.146407	0.116266
0.209306	0.237139	0.198193	0.225157	0.353137	0.406564	0.167479	0.123005
0.218647	0.238429	0.196870	0.215225			0.139926	0.134249
0.208781	0.233957	0.190754	0.210420			0.151721	0.139752
						0.146268	0.127280
						0.148384	0.140696
Pearson Correlation Coefficient		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**		**Pearson Correlation Coefficient**	
r=0.8950 p=0.0160 95% CI 0.3045-0.9885		r=0.8864 p=0.0186 95% CI 0.2666-0.9876		r=0.9819 p=0.0181 95% CI 0.3703-0.9996		r=-0.8502 p=0.0075 95% CI 0.3627-0.9723	

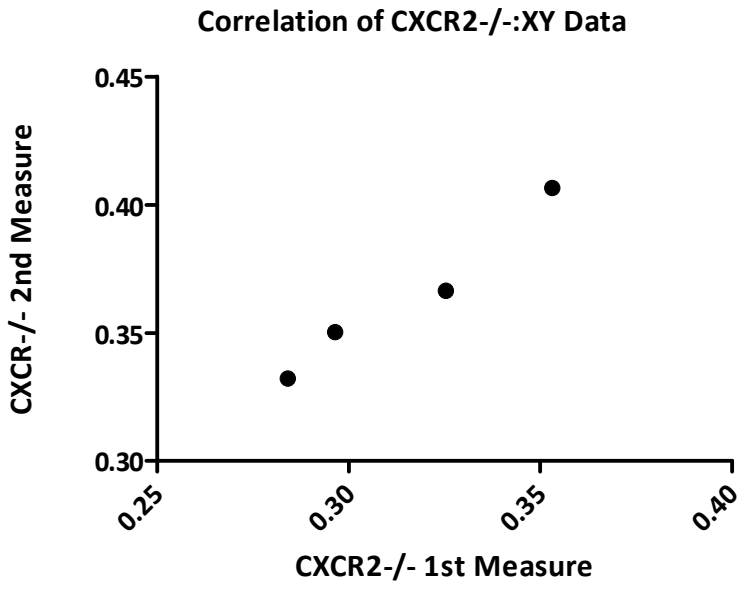
Balb/c bone loss data



Balb/c CNV bone loss data



CXCR2$^{-/-}$ bone loss data



CXCR2$^{-/-}$ CNV bone loss data